UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE EDUCATION
(301) 295-3913
FAX (301) 295-6772

APPROVAL SHEET

Title of Dissertation: "A Thapsigargin-Resistant Intracellular Calcium Sequestering Compartment in Rat Brain"

Name of Candidate: LCDR William D. Watson
Doctor of Philosophy Degree
31 March 2000

Dissertation and Abstract Approved:

Thomas Cote, Ph.D., Department of Pharmacology — 4-12-00 Date
Neuroscience Program
Committee Chairperson

Ajay Verma, M.D., Ph.D. — Date
Department of Neurology and Neuroscience Program
Committee Member

Brian Cox, Ph.D. — 4/12/2000 Date
Department of Pharmacology
and Neuroscience Program
Committee Member

He Li, M.D., Ph.D., Department of Psychiatry — 4-12-00 Date
and Neuroscience Program
Committee Member

Yoshitatsu Sei, M.D., Ph.D. — 4-12-00 Date
Department of Anesthesiology and Neuroscience Program
Committee Member

COPYRIGHT STATEMENT

The author hereby certifies that the use of any copyrighted material in the dissertation entitled:

A THAPSIGARGIN-INSENSITIVE INTRACELLULAR CALCIUM SEQUESTERING COMPARTMENT IN RAT BRAIN

is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

ABSTRACT

Title of Dissertation: A Thapsigargin-Insensitive Intracellular Calcium Sequestering Compartment in Rat Brain

William D. Watson, Ph.D., 2000

Thesis directed by: Ajay Verma, M.D./Ph.D., Assistant Professor Department of Neurology

Calcium plays a central regulatory role in the normal function of all cells. Electrical, secretory, and metabolic activities of cells in the brain require fine control over ionized cytoplasmic calcium levels. Intracellular calcium levels are controlled by a diverse set of cytoplasmic and membrane-associated mechanisms including calcium binding proteins, channels, pumps, and exchangers. The endoplasmic reticulum (ER) calcium stores have a major impact on neuronal intracellular signaling. Most of the ER in neurons and glia appears to accumulate calcium by energy driven ion pumps known as sarco/endoplasmic reticulum calcium ATPases (SERCAs), which are potently and selectively inhibited by thapsigargin. However, the ER represents a heterogeneous network of cisternae in which calcium-accumulating subcompartments may be spatially and functionally distinct. We describe here the characterization of a novel calcium accumulating subcompartment of rat brain ER, which is insensitive to thapsigargin. This compartment accumulates

calcium in a magnesium and ATP-dependent manner and is distinguished from thapsigargin-sensitive calcium pools with respect to anion permeability, inhibitor sensitivity, sensitivity to calcium mobilizers, and brain anatomical distribution.

A THAPSIGARGIN-INSENSITIVE INTRACELLULAR CALCIUM SEQUESTERING COMPARTMENT IN RAT BRAIN

by

William Doster Watson, M.D.

Thesis/dissertation submitted to the Faculty Committee of the Neuroscience Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy

2000

DEDICATION

This body of work is dedicated in large measure to the three people in my life: my wife, my mother, and my daughter. My wife, Michelle, has been a cornerstone of normalcy in an otherwise hectic seven years of dedicated study (medical school immediately followed by post graduate school). My mother, Jane, provided me with my deep commitment to learning and instilled in me the drive necessary to pursue my dreams. Finally, this work would not have been initiated or completed without the birth of my daughter, Mary Grace. She is an inspiration and undisguised daily blessing to us all.

ACKNOWLEDGEMENTS

I am forever indebted to my thesis advisor, Ajay Verma, for the person he is: a superb advisor, extraordinary scientist and human being. I absolutely could not have completed this thesis without his dedicated personal investment and technical expertise. If he achieves a mere fraction of the science revelations he has on a daily basis, he will have a career that is the envy of the modern world. Despite his endless and exhausting laboratory and clinical responsibilities, he has always anticipated and promptly attended to my needs extending to me courtesies far beyond advisor duties. His passion for discovery is highly infectious; his fund of scientific knowledge vast. He allowed me to make mistakes – and learn from them. Most importantly, he has provided me with a question-based, fundamental approach to science that I will utilize throughout my own career. I could not have asked for, nor received, a better thesis advisor.

I am grateful to my dissertation committee members for their time, personal support, and scientific advice: Dr. Tom Cote (Chairman), Dr. Brian Cox, Dr. Yoshi Sei, and Dr. He Li. All members were very accessible to me for questions and discussions, and they significantly contributed to the success of this project.

This dissertation would have been utterly impossible without the patient guidance and technical assistance of many laboratory personnel, including: Stephen Facchina, Dr. Peter Acs, Alison White, Dr. Andy Forbes, Dr. Susan Beckwith, Emily Clements, Jill Conover, and Lee Pitts. I have learned through

their efforts that science can be thought of as an apprenticeship. I will not forget their lessons, and I treasure their friendship. I am also thankful to Dean Ferrera and Arash Salardini, visiting students who contributed thought, energy, and humor towards completing this project.

I also thank collaborators who helped make this project possible: Dr. Regina Armstrong for providing primary astrocyte cultures and Dr. Aviva Symes for providing NBFL neuroblastoma cell cultures.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP.....Adenosine triphosphate

BHQ.....2,5-di-(*tert*-butyl)-1,4-benzohydroquinone

Ca^{2+} .....Calcium

CICR.....Calcium induced calcium release

cADPr.....Cyclic ADP ribose

CPA.....Cyclopiazonic acid

IP_3.....Inositol 1,4,5-triphosphate

IP_3R.....IP_3 receptor

NAADP.....Nicotinic acid-adenine dinucleotide phosphate

PMCA.....Plasma membrane calcium ATPase

RyR.....Ryanodine receptor

SERCA.....Sarco/endoplasmic reticulum calcium ATPase

SR/ER.....Sarco/endoplasmic reticulum

TG.....Thapsigargin

TG-I.....Thapsigargin-insensitive

TG-S.....Thapsigargin-sensitive

Van.....Vanadate

INTRODUCTION

Intracellular Ca^{2+} homeostasis is crucial for cell survival.

The importance of maintaining cell Ca^{2+} homeostasis is best exemplified by the consequences of disturbances in Ca^{2+} compartmentalization. The Ca^{2+} messenger system has a central role in mediating the contraction of all forms of muscle, secretion of exocrine, endocrine, and neurocrine products, regulation of glycogenolysis and gluconeogenesis, intracellular transport, secretion of fluids, and the growth and division of cells. Perturbation of the Ca^{2+} messenger system by disease and toxicants may place the regulation of such cellular functions outside the normal range of physiological control. Sustained increases in intracellular Ca^{2+} can also directly lead to cell death via uncontrolled activation of Ca^{2+} stimulated catabolic enzymes, such as proteases, lipases, and nucleases, and disruption of the cytoskeletal network [15, 69]. In addition, excessive cytosolic Ca^{2+} leads to massive uptake by the mitochondria resulting in formation of free radicals, increase in mitochondrial membrane permeability, and disturbances in oxidative phosphorylation [76]. Since the general role of calcium in cell death ("the calcium hypothesis") was first proposed over two decades ago [118], numerous studies have documented that nerve cells accumulate excessive Ca^{2+} when they exhibit cytopathology [60, 75]. The central concept

that disrupted Ca^{2+} homeostasis in cells leads to cell death makes research into the complex mechanisms that control internal Ca^{2+} content in physiological as well as diseased states essential.

Calcium is an intracellular second messenger with unique properties.

All cells contain elaborate systems for the spatial and temporal regulation of the Ca^{2+} ion, as well as biochemical response systems that are, in turn, regulated by changes in the concentration of intracellular Ca^{2+}. Soluble and membrane proteins decode the Ca^{2+} signal by complexing with it and changing conformations to mediate many basic cell functions, including gene expression, muscle contraction, cell growth and division, vesicular trafficking, secretion, and energy production. In resting cells, the cytoplasmic Ca^{2+} concentration is approximately 10^{-7}M [20]. This low resting concentration makes Ca^{2+} suitable as an intracellular messenger with small increases in concentration being equivalent to large fractional changes. Unlike other intracellular messengers such as cyclic AMP, cyclic GMP, diacylglycerol, phosphoinositides, nitric oxide, or carbon monoxide, Ca^{2+} is unique in that it cannot be readily created or destroyed. Therefore, the level of cytosolic Ca^{2+} is precisely controlled via its translocation across biological membranes. These mechanisms remove Ca^{2+} from the cytosol across the plasma membrane and into intracellular organelles and release Ca^{2+} into the cytosol again for signaling.

Rapid entry of extracellular Ca^{2+} into the cytosol occurs along its electrochemical gradient via voltage-gated, ligand-gated, and receptor operated channels. Calcium can exit the cell via ubiquitously expressed, outwardly directed plasma membrane Ca^{2+}-ATPases (PMCAs), as well as Na^{+}/Ca^{2+}-exchangers found typically in excitable tissues. The two best understood intracellular compartments participating in Ca^{2+} sequestration are the mitochondria and the sarcoplasmic (SR) or endoplasmic reticulum (ER). Accumulation of Ca^{2+} into the mitochondrial matrix is powered by the electrochemical potential across the inner mitochondrial membrane. Under physiologic conditions, calcium entry into mitochondria may activate matrix enzymes critical to mitochondrial energy production [145]. Excessive Ca^{2+} accumulation during cell injury, however, may destroy mitochondrial function. The saturation of mitochondrial Ca^{2+} sequestering ability represents a key step in necrotic cell death [76, 77]. The SR and ER contain calcium/magnesium ATPases, called SERCAs (sarco-endoplasmic reticulum calcium atpases), for rapid, energy dependent sequestration of Ca^{2+}. Their efficient uptake activity enables the SR-ER to act as a Ca^{2+} sink for excess cytosolic Ca^{2+} and as a source of stored Ca^{2+} that can be released upon stimulation by various second messengers. For signal-mediated release, the SR and ER contain second messenger-sensitive Ca^{2+} release channels, which allow for rapid increases of cytosolic ionized Ca^{2+} levels to be initiated by hormone and neurotransmitter receptor binding [26, 65, 73, 122, 123, 136].

Calcium release from SR-ER pools is a major event in signal transduction.

One of the principle messenger systems that influences intracellular Ca^{2+} is the phosphoinositide cycle in which hydrolysis of phosphatidylinositol - bisphosphate by receptor mediated activation of phospholipase C forms the second messenger, inositol 1,4,5-trisphosphate (IP_3) at the plasma membrane [13, 24, 41]. Diffusable IP_3 mobilizes Ca^{2+} from ER stores via a specific receptor channel protein, which has been localized in the brain by autoradiography [141, 145] and immunochemistry [114], purified [128], functionally reconstituted [37], and cloned [42]. This protein exhibits regionally heterogeneous anatomical distribution in the brain and appears to be localized within cells to a subset of the ER at the electron microscopic level [114, 121].

In addition to Ca^{2+} release stimulated by IP_3 binding to IP_3 receptor (IP_3R) channels, the SR-ER released Ca^{2+} can be stimulated by Ca^{2+} itself in a process referred to as Ca^{2+}-induced Ca^{2+} release (CICR) [20, 26, 56, 123, 138]. During CICR, Ca^{2+} enters the cytoplasm through plasma membrane channels or IP_3Rs and triggers Ca^{2+} release in a feed forward process primarily mediated by the ryanodine receptor (RyR) channel [3, 8, 26, 40, 59, 81, 82, 122]. Ryanodine is a plant alkaloid, which binds tightly to the SR-ER RyR channel and holds it in an open state [63]. Experimentally, the RyR channel can be opened by millimolar levels of caffeine and several toxins, including ryanodine and cardiotoxic anthraquinone doxorubicin [106]. There is no universally proven endogenous second messenger for RyRs other than Ca^{2+} itself. However, the

endogenous metabolite cyclic ADP ribose (cADPr), a newly described second messenger [43, 80], has been shown to enhance CICR via the RyR channel in some permeabilized cells [64] and may serve this role more generally. Recently, other endogenous Ca^{2+} releasers have also been reported. These mobilizers, which include long chain fatty acids [50], coenzyme A derivatives [38], and oleoyl lyphosphatidic acid [143], may represent novel second messengers that apparently cause Ca^{2+} release from internal pools. The actual tissue specificity, sub-compartments, as well as release channels that these new mobilizers act upon remain unknown.

Understanding how the RyRs and the IP_3Rs interact within cells to control cytoplasmic Ca^{2+} concentrations has been challenging. Do these SR-ER receptor channels release Ca^{2+} from the same or different Ca^{2+} pools? Using a novel autoradiographical procedure employing radiotracer $^{45}Ca^{2+}$, Verma *et al.* found that the location of ryanodine-sensitive Ca^{2+} pools matched caffeine-sensitive Ca^{2+} pools as well as ^{3}H-labeled ryanodine binding sites in the brain [140]. They also localized IP_3-sensitive pools finding that their regional localization in brain is predominantly complementary to that of the RyRs [140]. Furthermore, the intracellular expression of these receptor-channels in neuronal ER is also reciprocal with RyRs located primarily in the soma [120] and IP_3Rs predominantly in the periphery [89, 97]. Thus, the expression of intracellular Ca^{2+} release channels is regionally as well as sub-cellularly heterogeneous in the brain. The specialization of SR-ER compartments with respect to the

spatially distinct expression of these two receptor-channels may have profound functional implications. It has been suggested that the more central RyRs function to amplify sub-maximal Ca^{2+} release signals initiated by stimulated peripheral IP_3Rs [57]. The IP_3R-gated and CICR mechanisms have been observed to activate sequentially [120], which may be essential to the generation of cytoplasmic Ca^{2+} waves or oscillations that have been documented in many cell types [10, 12, 95, 120, 127]. These cytosolic Ca^{2+} oscillations are thought to represent an intracellular digital signal with significant biological information being encoded by the oscillation frequency and amplitude characteristics [10, 11, 96]. In this fashion, it is possible that locally initiated Ca^{2+} release signals produced in the cell periphery via the IP_3R may be amplified and broadcast to distant regions, including the nucleus, via the RyR.

Ion motive ATPases power the removal of calcium from the cytosol.

The rapid removal of Ca^{2+} from the cytosol is as important to its messenger role as is its release. Thus, following Ca^{2+} activated contraction, muscle relaxation requires the ATP-dependent removal of Ca^{2+} from the cytosol. Each heartbeat, therefore, involves cyclic Ca^{2+} release into the cytosol of cardiomyocytes followed by its removal. The ATP-driven ion-motive enzymes (referred to as ion-motive ATPases) involved in this process, are believed to have originally evolved to prevent cell swelling from the influx of extracellular salts and water across the semi-permeable plasma membrane [88]. This reasoning reflects the fact that many ion-specific ATPases resemble those

selective for Ca^{2+}, and all eukaryotic ATP-driven pumps possess prokaryotic counterparts. Ion-motive ATPases, in general, couple the free energy of ATP-hydrolysis or synthesis to the translocation of ions across membranes and can be grouped as V-, F-, and P-types [109]. Ion-motive ATPases of the V-type are those associated with membrane-bound organelles other than the ER and mitochondria, i.e., yeast vacuoles or secretory vesicles. These V-type ATPases may be involved in vesicle acidification, and can be specifically inhibited by the drug bafilomycin. The F-type ATPase class includes the F_1F_0 types found in bacteria, chloroplasts, and mitochondria [4, 53, 119]. The mitochondrial F_1F_0 ATPase is an H^+-pump that is generally thought of as an ATP synthase, but it can operate in either direction. It can be selectively inhibited by azide or oligomycin. ATPases of the P-type form covalent phosphorylated intermediates as part of their characteristic reaction cycle after cations bind the pump at specific high affinity sites [29, 47].

Eukaryotic cells express many types of P-type ATPases, such as the Na^+/K^+, K^+/H^+, and Ca^{2+} pumps in the plasma membrane. Vanadate, an analog of phosphate, is a general inhibitor of P-type ATPases and helps distinguish the P-types from the V- or F-types [86]. Several other highly selective inhibitors have been discovered which act on specific P-type ATPases. For example, ouabain is an exclusive inhibitor of the Na^+/K^+-ATPase [1, 91], while omeprazole only blocks the activity of the K^+/H^+-ATPase [2, 9]. The plasma membrane Ca^{2+}-ATPases (PMCA) are Mg^{2+}-dependent Ca^{2+}-ATPases ubiquitously expressed in

eukaryotic cells [20], with the human pump being the product of a multigene family whose primary transcripts are subject to developmental and tissue-specific alternative processing. The PMCA is a P-type ATPase that is specifically targeted for the plasma membrane and exports Ca^{2+} from the cytoplasm. Eosin and carboxyeosin have been touted as inhibitors of the PMCAs, but their effects have yet to be thoroughly examined on all P-type ATPases [23, 125].

SERCAs: a family of P-type ATPases involved in ER Ca^{2+} pumping.

Like the PMCAs, the SERCAs are a specialized family of P-type ATPases. These Mg^{2+}-dependent Ca^{2+}-ATPases are found in the SR-ER of all mammalian cells and are essential to maintaining low cytoplasmic resting Ca^{2+} content. SERCAs undergo a reaction cycle requiring ATP hydrolysis and phospho-enzyme intermediate formation to transport Ca^{2+} from the cytoplasm into the SR-ER lumen.

The SERCAs are encoded by at least three separate genes located on different chromosomes [33]. Different SERCA isoforms are synthesized via alternative gene splicing mechanisms regulated by development and tissue location. The resulting isoforms vary at the C-terminus. All SERCAs encode a cytoplasmic region containing a catalytic site and transmembrane domains that form a channel-like structure, which permits Ca^{2+} translocation across the membrane [6, 25, 62, 142]. Although functional differences between isoforms

may exist, as best exemplified by SERCA2b having a much higher affinity for Ca^{2+} than SERCA2a [137], the reason for SERCA isoform diversity is not known. The SERCA2b isoform is expressed ubiquitously and is the predominant form in the brain [16, 84, 98]. The SERCA3 pump is also found in brain, as well as in other non-muscle tissues [18, 146, 147]. The SERCA enzymes, in general, function as monomers (~100kD mol. wt.) [5]. Much of what is known about the relationship of SERCA function and cell physiology has been learned in heart muscle, where relaxation following contraction is the result of rapid uptake of myoplasmic Ca^{2+} by the SERCA2a isoform [84]. Transgenically induced SERCA overexpression in cardiac myofibrils improves heart performance by increasing the relaxation rate through quicker Ca^{2+} uptake from the myoplasm and the availability of SR-stored Ca^{2+} for subsequent release [31].

Thapsigargin is a specific tool for studying intracellular Ca^{2+} pools and SERCA function.

A valuable experimental tool for exploring intracellular Ca^{2+} was introduced with the discovery of thapsigargin (TG). This tumor promoting sesquiterpene lactone, derived from the plant *Thapsia garganica*, interferes with intracellular Ca^{2+} regulation by blocking internal mechanisms of ER Ca^{2+} sequestration [132]. More specifically, TG potently inhibits all known SERCA isoforms with low nanomolar K_i values [19, 84, 104]. This inhibition is selective and does not affect the activity of plasma membrane Ca^{2+}-ATPases (PMCAs) or

any other types of ion pumps [84]. Upon binding to SERCAs in the Ca^{2+} dissociated conformation, TG irreversibly blocks the ability of the enzyme to bind Ca^{2+} [115]. Recently, site-directed mutagenic studies identified a short, six amino acid region of the S3 stalk segment, that dramatically reduces the TG sensitivity of SERCAs when mutated [151]. In fact, the single mutation of Gly257 to Ile alone is effective in reducing the sensitivity to TG by 1000-fold [151].

The potent, selective blockade of Ca^{2+} accumulation in the ER by TG has made it possible to study the significance of ER Ca^{2+} pools to cell function and growth. Intralumenal Ca^{2+} content controls several essential ER functions including the translation of mRNA and the processing, folding, and assembly of proteins [78, 99, 103, 117]. Brief exposure of cultured cells to nanomolar doses of TG has been shown to cause prolonged emptying of Ca^{2+} pools, inhibition of protein synthesis [87, 144] and growth arrest [46], while prolonged exposure causes cell death (for review see [133]). Cyclopiazonic acid (CPA), a fungal toxin, and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ) are other selective inhibitors of SERCAs that are reversible and are chemically unrelated to TG [34, 36, 90, 108].

TG has also been used recently to investigate the importance of lumenal ER Ca^{2+} levels in triggering the apoptotic death of neuronal and non-neuronal cells [14, 67, 100, 135, 152]. Thapsigargin treatment of cells causes an acute

rise in cytosolic Ca^{2+} along with depletion of ER pools. Several independent reports now suggest that the depletion of ER Ca^{2+} pools triggers apoptosis in TG-treated cells [14, 55, 100, 111]. How depletion of ER Ca^{2+} triggers apoptosis is not known, but recent evidence points to a link with the bcl family of apoptotic and antiapoptotic proteins. Cells induced to overexpress the endogenous anti-apoptotic protein Bcl-2 are insensitive to apoptosis triggered by TG. Furthermore, the ER of cells overexpressing transfected Bcl-2 has been reported to become insensitive to TG-inhibition [79]. Thus, the experimental use of TG has provided new insight into cell processes involving calcium, including the importance of maintaining ER Ca^{2+} levels to help prevent apoptosis.

Novel ER-like compartments accumulate Ca^{2+} in the presence of thapsigargin (TG).

Recently, the use of TG revealed what appears to be a novel, non-mitochondrial intracellular Ca^{2+} sequestering compartment. This compartment was originally described in brain microsomal preparations, which lacked mitochondria [140]. In this preparation TG can only inhibit 85 - 90% of the net ATP-dependent Ca^{2+} accumulation while the calcium ionophore A23187 is 100% effective [140]. Thus, 10-15% of net Ca^{2+} uptake into brain microsomal compartments is mediated by a TG-insensitive (TG-I), ATP requiring mechanism. Since this original description, the TG-I Ca^{2+} compartment or pool has been described only to a limited extent in cell culture studies, with very little work having been done in tissue preparations. Table 1 displays a current survey

of the literature regarding information about the TG-I pool, such as Mg^{2+} and ATP-dependence, the effects of Ca^{2+} releasing agents, and suggested cell functions of this pool. From the studies outlined in Table 1, few consistent or universal characteristics of the TG-I Ca^{2+} compartment(s) appear to emerge. For instance, the TG-I Ca^{2+} pool is IP_3-sensitive in some cell lines, but not in others. Also, Ca^{2+}-induced Ca^{2+} release (CICR) is suggested as a function for the TG-I store in some studies, but has been specifically discounted in sea urchin homogenates. Vanadate was found to effectively inhibit TG-I Ca^{2+} uptake in a mutant cell line, DC-3F/TG2, and in pig brain cell homogenates, which may indicate that the TG-I Ca^{2+} transport mechanism is a P-type ATPase. However, vanadate sensitivity is not reported in other studies. Likewise, it is unclear whether or not BHQ and CPA have any effect on the TG-I Ca^{2+} pool. CPA inhibited TG-I Ca^{2+} uptake in rat ventricle isolates and pig brain homogenates, but it had no effect on the TG-I pool in other studies. One reason for this inconsistent picture of the TG-I pool may be the predominant use of cancer cell lines in studies performed so far. Cancer cells may have unusual adaptations (for example, alterations in the apoptotic response or Bcl-2 overexpression) affecting their Ca^{2+} homeostasis, which may confound interpretations regarding TG-I pool function in normal tissues. Clearly, much remains to be understood regarding the function, anatomical distribution, uptake mechanism, and regulation of the TG-I Ca^{2+} pool in normal tissues.

Is the TG-I Ca^{2+} pool segregated anatomically in the brain as are IP_3-sensitive and caffeine-sensitive Ca^{2+} pools? Initial microsomal studies reported regional differences in the activity of the TG-I Ca^{2+} pool in rat brain with the thalamus and spinal cord having a greater proportion than the cerebellum or olfactory bulb [140]. Differential expression of Ca^{2+} pools may have significant implications for the regional vulnerability of the brain to insults, which produce cellular Ca^{2+} overload. The mammalian hippocampus displays unique regional sensitivity to different types of insults with the CA-1 subfield being extremely sensitive to hypoxic-ischemic insults [66, 68, 71, 94, 129]. Verma *et al.*, utilizing a novel, *in situ* technique to autoradiographically visualize ATP-dependent $^{45}Ca^{2+}$ transport into fresh-frozen rat brain sections, demonstrated that the CA-1 region is enriched in IP_3-sensitive calcium pools [140]. SERCA immunoreactivity in this region is relatively low [121]. This uneven relationship in ER Ca^{2+} release channels and uptake pumps in the CA-1 subfield provides a basis for pathological vulnerability to ER Ca^{2+} store depletion and elevated cytoplasmic Ca^{2+}. Similarly, discretely localizing the TG-I Ca^{2+} pool may eventually help explain why some regions are more vulnerable to certain types of insults than other regions.

Is the TG-I Ca^{2+} pool loaded by a novel SERCA-like Ca^{2+}-ATPase that is TG resistant? As mentioned previously, mutations in the SERCA S3 stalk segment dramatically reduces its TG sensitivity [151]. A differential splice mechanism, thus, could specifically alter this region and cause known SERCA

isoforms to become TG-I. The DC-3F Chinese hamster lung cell line was made resistant to TG by long-term, step-wise exposure to increasing doses of TG [49]. These cells resemble the parent cells but display a marked increase in the level of a TG-I ER calcium pool. These adaptive changes suggest that the TG-I Ca^{2+} sequestering mechanism can be selectively upregulated and is capable of sustaining normal cell growth and physiology in the absence of the prominent TG-sensitive (TG-S) ER Ca^{2+} uptake. Recently, an intracellular SERCA-like Ca^{2+}/Mg^{2+}-ATPase insensitive to TG inhibition has been isolated and cloned from the ciliated protozoan, *Paramecium tetraurelia* [54, 72]. This new SERCA isoform apparently lacks binding sites for TG and shows a unique intracellular distribution, being confined to subplasmalemmal "alveolar sacs" which are believed to play a role in stimulus-secretion coupling in this organism [54]. This demonstrates that at least one TG-I SERCA-like ATPase is expressed normally in simple eukaryotes and suggests that similar enzymes may participate in eukaryotic Ca^{2+} homeostasis more universally. It is entirely possible, however, that the observed TG-I activity is accounted for by a Ca^{2+} sequestering mechanism not involving a Ca^{2+} specific ion-motive pump. For example, ATP-dependent transport of another ion such as H^+ with subsequent counter ion exchange for Ca^{2+} may be responsible for Ca^{2+} accumulation within cellular compartments. Elucidating the mechanism by which the TG-I pool accumulates Ca^{2+} in vertebrates may greatly advance our understanding of eukaryotic Ca^{2+}regulation by either identifying new SERCA-like activities or novel Ca^{2+} transport mechanisms.

The TG-I calcium pool may represent a unique intracellular compartment with distinct second messenger sensitivities and anion permeability characteristics.

The net flux of Ca^{2+} into the SR-ER lumen depends on other factors in addition to SERCA activity. The accumulation of $^{45}Ca^{2+}$ into the ER lumen can be completely inhibited using the calcium ionophore A23187, which has no effect on the enzymatic uptake activity of the SERCAs. A23187 is a lipid-soluble compound that inserts itself into the walls of membrane-bound compartments and acts like a revolving door to prevent the accumulation of Ca^{2+} on either side. Thus, leak pathways for Ca^{2+} will reduce its net accumulation within the lumen of membranous compartments. Similarly, whether IP_3R or the RyR release channels are in the closed or open state will directly influence the net Ca^{2+} accumulation in isolated ER preparations. The Ca^{2+} release channels and SERCAs are distinct proteins with unique expression patterns and may be differentially represented in distinct Ca^{2+} buffering compartments. Thus, the functional Ca^{2+} flux properties of ER compartments can be selectively sculpted in different brain cells via differential expression of ER pumps and channels. In rat brain microsomes IP_3-sensitive Ca^{2+} compartments were shown to represent only a subset of the thapsigargin-sensitive (TG-S) compartment, and the TG-I compartment was found to be insensitive to IP_3 [140]. This selective sensitivity of the TG-S and TG-I Ca^{2+} uptake to IP_3 mobilization suggests that the TG-S

and TG-I pools represent unique, non-contiguous compartments with distinct uptake and release mechanisms.

The ER is also permeable to anions, and the co-transport of anions with Ca^{2+} is believed to facilitate overall Ca^{2+} flux by charge neutralization. Anion transport along with Ca^{2+} may help avoid the buildup of an opposing electrical gradient to further Ca^{2+} uptake. By reducing intra-lumenal free calcium concentration, anions may also decrease feedback inhibition of Ca^{2+} on SERCA activity [35]. Phosphate and oxalate are two permeable anions that strongly stimulate Ca^{2+} uptake *in vitro* by forming intra-lumenal salts with the actively transported Ca^{2+} [52, 61]. These anions penetrate ER vesicle membranes through poorly defined channel proteins [126], which may themselves be differentially expressed with respect to Ca^{2+} pumps and release channels. A model to help explain net Ca^{2+} flux into a Ca^{2+} accumulating compartment is shown with representative membrane-bound proteins for uptake, anion entry, passive efflux and receptor channel Ca^{2+} release (Figure 1). The IP_3-sensitive Ca^{2+} pool has been shown to be permeable to oxalate, but it is not known whether the TG-S and TG-I Ca^{2+} compartments also have distinct anion permeabilities. Does the TG-I Ca^{2+} pool distinguish a unique compartment or organelle with unique anion permeability and release channel properties? Which, if any, second messengers release Ca^{2+} from the TG-I pool? Our appreciation of the overall dynamics of Ca^{2+} homeostasis appears far from complete and a better understanding of TG-I Ca^{2+} sequestration may provide a clearer picture.

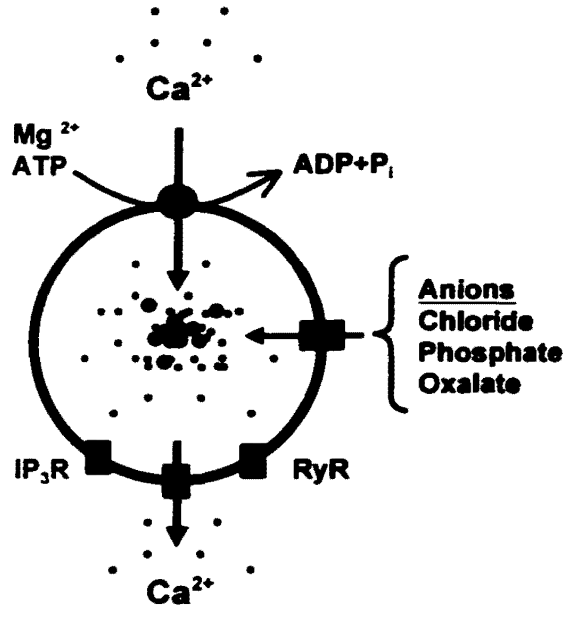


Figure 1. Model of Ca^{2+} flux in ER- or SR-like vesicles. Ca^{2+} specific Mg^{2+} ATPase activity (filled circle) transports Ca^{2+} uni-directionally into the vesicle lumen. Anion co-transport, which neutralizes the accumulated charge, occurs via poorly defined anion channels. When oxalate or phosphate is present in the assay, their co-accumulation leads to insoluble complexation with Ca^{2+}. Ca^{2+} release pathways are also found in ER or SR vesicles which allow leak of Ca^{2+} out from the lumen. These include a poorly defined, non-specific Ca^{2+} leak path as well as specific families of second messenger activated Ca^{2+} release channels, such as the inositol triphosphate receptor (IP3R) and the ryanodine receptor (RyR).

Summary

Loss of Ca^{2+} compartmentalization is a key event that can lead to cell death. Both necrotic and apoptotic cell death are dependent on changes in cellular Ca^{2+} compartmentalization. Calcium acts as a universal second messenger in cells being involved in the control of muscle contraction, cell growth and division, secretion, chemotaxis, and apoptosis. Regulation of Ca^{2+} compartmentalization by intracellular pumps and release channels is essential for it to be an effective second messenger. The ER is a major intracellular Ca^{2+} buffering organelle, which may show regional specialization within a cell and

among cells. Ca^{2+} uptake and release properties of the ER represent the actions of several unique proteins. Ca^{2+} is sequestered into the ER (or SR) by P-type Ca^{2+}/Mg^{2+}-ATPases called SERCAs, which are encoded by three separate differentially expressed genes. The experimental use of thapsigargin (TG), a potent universal inhibitor of eukaryotic SERCAs, has revealed what appears to be a novel, non-mitochondrial intracellular Ca^{2+} sequestering compartment in cell cultures and tissue homogenates.

Despite a decade of research since the discovery of thapsigargin-insensitive (TG-I) Ca^{2+} uptake, little more has been learned about it. Microsomal Ca^{2+} uptake in the presence of TG appears to represent the activity of a novel non-mitochondrial compartment. How much of an overall contribution the TG-I compartment makes to brain intracellular Ca^{2+} buffering remains unknown. Does the TG-I uptake mechanism have different sensitivities to ATP and Mg^{2+} than the TG-S mechanism? Is a P-type ion-motive ATPase involved in loading the TG-I Ca^{2+} pool? Do Ca^{2+} precipitating anions stimulate uptake into the TG-I Ca^{2+} compartment? Does TG-I Ca^{2+} uptake display discrete regional heterogeneity in rat brain? Our preliminary experiments have addressed some of these key questions about the TG-I Ca^{2+} pool.

TABLE 1. Summary of published information to date regarding a novel thapsigargin-insensitive (TG-I) intracellular Ca^{2+} buffering compartment.

Source	ATP/Mg-depend	Method of Ca^{2+} Monitor	Sensitivity to Other SERCA Inhibitors	IP_3-Sensitive Release	CICR Modulated release	Modulation by Other Ca^{2+} Regulators	Proposed F(x) of TG-I Pool	Reference
Rat brain microsomes	+/+	$^{45}Ca^{2+}$		No				Verma et al. BBRC 1990; 172(2):811-6
Rat salivary acinar cells				Yes		Caffeine	Ca^{2+} oscillations	Foskett and Wong JBC 1991 Aug 05; 266(22): 14535-8
GH4C1 cell line	+/+	Fluo3		No	Yes	Caffeine	Ca^{2+} oscillations	Tanaka and Tashjian Biochem 1993 Nov 16; 32(45): 12062-73
DC-3F/TG2 cell line	+/+	$^{45}Ca^{2+}$	Vanadate	Yes			Mutant cell growth	Waldron *et al.* JBC 1995 May 19; 270(20): 11955-61
Human neutrophils		Fura2		No		Polyenoic very-long-chain fatty acids (> 22 C's)	Superoxide production; release of intracell Ca^{2+}	Hardy et al. Biochem J 1995; 811:689-697
Dog mesenteric art & Rat *vas deferens* smooth muscle	+/	$^{45}Ca^{2+}$						Darby *et al.* Can J Physiol Pharmacol 1996 Feb; 74(2): 182-92
Sea urchin egg homogenates	+/+	Fluo3		No	No	Nicotinic acid-adenine dinucleotide phosphate (NAADP)	Unlikely to be involved in CICR	Genazzani and Galione 1996; 315:721-725
DC-3F/TG2 cell line		Fura2		Yes		Oleoyl lysophosphatidic acid	Mutant cell growth	Waldron *et al.* JBC 1997 Mar 7; 272(10): 6440-7
Rat pancreatic β-cells		Indol 1				Tetracaine	RyR-stimulated release	Brodin *et al.* Eur J Pharmacol 1997 Mar 25; 327:257-62
Rat cortical glia	+/							Simpson and Russell Biochem J 1997 Jul 1; 325(2): 272-8

TABLE 1. Summary of published information to date regarding a novel thapsigargin-insensitive intracellular Ca^{2+} buffering compartment (cont.).

Source	ATP/Mg-depend	Method of Ca^{2+} Monitor	Sensitivity to Other SERCA Inhibitors	IP_3-Sensitive Release	CICR Modulated release	Modulation by Other Ca^{2+} Regulators	Proposed F(x) of TG-I Pool	Reference
Rat hepatocytes							Ca^{2+}-ATPase in transit to plasma membrane	Taylor et al. Mol Biol Cell 1997 Oct; 8(10): 1911-31
Rat pancreatic acinar cells		Fura2		No		Palmitoyl CoA	CICR-stimulated release	Fitzsimmons *et al.*. JBC 1997 Dec 12; 272(50): 31435-40
Pig brain membrane isolates	+/		Vanadate CPA					Salvador and Mata Arch Biochem Biophys 1998 Mar 15; 351(2): 272-8
Dog salivary gland cells				Yes	Yes	cADP-ribose (calmodulin potentiates)		Yamaki *et al.*. J Dent Res 1998 Oct; 77(10): 1807-16
Rat ventricle isolates	+/+	$^{45}Ca^{2+}$	CPA		Yes	Ryanodine		Feher *et al.* Mol Cell Biochem 1998 Dec; 189(1-2): 9-17
Rat & Mouse pancreatic beta-cells	+/+	Indo-1				Tetracaine	Insulin secretion	Mears *et al.* Cell Calcium 1999 Jan; 25(1): 59-68
GH3 cells	+/	$^{45}Ca^{2+}$		No	Yes	Caffeine	Ca^{2+} oscillations	Hirono *et al.* Cell Calcium 1999 Feb; 25(2): 125-35

SPECIFIC AIMS

We proposed to understand the mechanism of uptake, pharmacological sensitivities to calcium mobilizers, and distribution of the TG-I Ca^{2+} pool in rat brain. The research plan for each of these is detailed below:

Specific Aim 1: Determine whether or not a Mg^{2+}-dependent, Ca^{2+}-activated ATPase activity involving a phospho-enzyme intermediate sequesters Ca^{2+} in the TG-I pool.

The objective of this specific aim was to characterize the mechanism involved in TG-I Ca^{2+} uptake in rat brain. TG-I Ca^{2+} accumulation was originally reported in brain microsomes [140], and it is possible that the TG-I uptake mechanism identified in isolated ER is structurally and functionally similar to SERCAs that also reside in the ER. However, the demonstrated ability to accumulate Ca^{2+} in microsomes in the presence of TG is insufficient evidence to classify the TG-I Ca^{2+} uptake mechanism as a Ca^{2+}-ATPase, much less as a SERCA-like Ca^{2+}-ATPase. The TG-I Ca^{2+} uptake mechanism may represent a previously unappreciated ion exchange mechanism. Such mechanisms are ubiquitous in cells and can accumulate Ca^{2+} through antiporter activity driven by the efflux potential of a different cation created by its own ion-motive ATPase. For

example, secretory vesicles require lumenal Ca^{2+} for enzymatic processes. Secretory vesicles have V-type H^+-ATPases, which concentrate H^+ into their lumens making them acidified. High lumenal H^+ can then exit vesicles along its electrochemical gradient through an H^+/Ca^{2+}-antiporter in exchange specifically for Ca^{2+} entry. This mechanism of Ca^{2+} uptake involves no Ca^{2+}-ATPase or SERCA activity and, therefore, would be insensitive to TG inhibition. However, whether or not this particular TG-I Ca^{2+} uptake mechanism contributes to the TG-I Ca^{2+} pool observed in rat brain tissue preparations is unknown. Our tissue preparations alone do not reliably discount the contribution of artifactual vesicles to TG-I Ca^{2+} uptake, such as plasma membrane vesicles. Other organelles besides the ER may contribute to TG-I Ca^{2+} uptake, which also could account for the increased proportional TG-I accumulation that we have observed in brain homogenates relative to isolated ER preps. Several organelles, such as mitochondria and the Golgi apparatus, contain lumenal Ca^{2+} and are thought to sequester calcium primarily through known ion exchange mechanisms. To exclude each of these organelles, which may contaminate our isolated ER preparations, we utilized a combination of organelle-specific ion-motive pump inhibitors and ionophores to identify their TG-I Ca^{2+} pool contribution in brain microsomes. Alternatively, the TG-I Ca^{2+} pool may represent the activity of a novel Ca^{2+}-ATPase that is "SERCA-like". In this case, the putative Ca^{2+} pump may be a P-type pump of the SERCA or the PMCA family. Along with vanadate inhibition of uptake activity, any P-type ATPase should exhibit an ion-sensitive (Ca^{2+} in this case) phospho-enzyme intermediate formation.

Section Objectives:

Objectives of this specific aim were: **(a)** to determine whether the TG-I uptake mechanism displays similar requirements for Ca^{2+}, Mg^{2+}, and ATP as SERCA, **(b)** to determine whether ion exchange mechanisms are utilized to transport calcium into the TG-I pool, and **(c)** to determine whether a Ca^{2+}-stimulated ATPase activity is associated with TG-I Ca^{2+} accumulation.

Plan:

The strategy for section objective (a) was to assess the dependence of the TG-I mechanism on ATP, Mg^{2+}, temperature, and redox modification in comparison to classical SERCA activity. Objective (b) was analyzed by measuring the effects of specific pump inhibitors and ionophores on TG-I Ca^{2+} uptake in whole brain microsomes. Objective (c) was determined by monitoring Ca^{2+}-sensitive ATPase activity and the formation of a phosphorylated enzyme intermediate in the presence of TG.

Rationale:

SERCAs are P-type Ca^{2+} pumps resident in the ER and are dependent on the presence of ATP, Mg^{2+}, and Ca^{2+} for activity. Functional (and structural) characteristics of the TG-I mechanism can be assessed in comparison to that of SERCAs by evaluating for similar dependency. Preliminary studies utilizing vanadate implicated the activity of a P-type ATPase in TG-I Ca^{2+} accumulation.

However, this fact does not exclude an ion exchange mechanism or prove that ATPase activity is Ca^{2+}-activated. At the completion of this aim, we provide evidence that the TG-I Ca^{2+} uptake mechanism in brain microsomes may utilize a P-type ATPase similarly sensitive to ATP, Mg^{2+}, and Ca^{2+}, but uniquely insensitive to reduction of sulfhydryl groups as compared to SERCA activity.

Specific Aim 2: Determine whether or not known intracellular Ca^{2+} store mobilizers affect the Ca^{2+} pool accumulated in the TG-I store.

An important step in determining whether the TG-I Ca^{2+} pool is functionally significant is to determine whether or not known modulators of intracellular calcium release channels can liberate its sequestered Ca^{2+}. While some studies have found that the TG-I calcium pool can be mobilized by agents acting at the IP_3R and RyR in cultured cancer cell lines, others have found no such sensitivities. We determined the ability of the calcium-mobilizing agents IP_3, caffeine, cADPr, and NAADP to release Ca^{2+} from the TG-I Ca^{2+} compartments in rat brain microsomes.

Section Objective:

The objective was to determine whether known Ca^{2+} mobilizing agents stimulate Ca^{2+} release from the TG-I Ca^{2+} pool in rat brain microsomes.

Plan:

The strategy to accomplish this section objective was to monitor microsome $^{45}Ca^{2+}$ accumulation in the presence of TG and several Ca^{2+} mobilizers: IP_3, caffeine, cADPr, and NAADP.

Rationale:

The ability of potential mobilizers to stimulate Ca^{2+} release from compartments filled by the TG-I Ca^{2+} uptake in rat brain is unknown. Since a subset of compartments in ER preparations identified by SERCA activity are stimulated by IP_3 and thus, express IP_3Rs, the TG-I Ca^{2+} uptake compartment may also be associated with specific receptor-channels. Determination of the sensitivity to agents that stimulate Ca^{2+} release from the TG-I pool will not only further define this compartment in terms of protein expression, but also provide clues about its functional role within intact cells in the brain.

<u>Specific Aim 3:</u> Determine the regional and cellular distribution of the thapsigargin-sensitive (TG-S) and thapsigargin-insensitive (TG-I) Ca^{2+} compartments in rat brain.

Many studies show that the TG-I Ca^{2+} pool is an intracellular compartment that contributes significantly to total Ca^{2+} buffering in different cell lines. However,

which brain cell types participate in TG-I Ca^{2+} uptake is not known. The regional anatomical distribution of the TG-I Ca^{2+} pool also remains to be elucidated.

Section Objectives:

To address this aim, we endeavored: **(a)** to determine the relative contribution of different types of brain cells, neurons or astrocytes, to TG-I Ca^{2+} uptake, and **(b)** to determine the regional anatomical distribution of TG-I Ca^{2+} uptake in rat brain.

Plan:

The strategy for section objective (a) was to measure Ca^{2+} uptake in the absence and presence of TG into permeabilized primary cultures of cerebral granular neurons and rat whole brain astrocytes. Objective (b) was addressed by analyzing $^{45}Ca^{2+}$ uptake in coronal and sagittal rat brain fresh frozen tissue sections via autoradiography.

Rationale:

It is known that specific brain regions and cell types are acutely sensitive to calcium-mediated injury. Consequently, understanding the regional and cellular distribution of TG-I Ca^{2+} uptake may be valuable in understanding regional brain vulnerability to certain insults. Collectively, the studies in this aim delineated the regional and cellular distribution of TG-I Ca^{2+} accumulation in normal rat brain.

MATERIALS AND METHODS

Materials

Reagents:

All reagents used in general laboratory procedures were purchased from Sigma Chemical Company (St. Louis, MO). Thapsigargin was obtained from Molecular Probes (Eugene, OR). [$^{45}Ca^{2+}$]-$CaCl_2$ and [γP^{32}]-ATP were obtained from NEN (Boston MA). All other reagents used were of the highest grade available.

Equipment:

Electrophoresis equipment: Novex, Inc. (San Diego, CA). Spectrophotometer: Hewlett Packard model 8453 (Rockville, MD). Fluorimeter/Luminometer: Perkin Elmer LS50B (Norwalk, CT). Ca^{2+}-specific electrode: SensorLink pH/ISE electrode (Orion Research, Inc., Charlestown, MA). Film developer: Kodak M35A X-omat Processor (Rochester, NY). Cryostat: Zeiss model HM505E Microm (Thornwood, NY).

Methods

Cell Cultures and Harvesting

Granule cells were dissociated from cerebella of 8-day old Wistar rat pups as described [32, 44, 101]. Cells were plated on poly-D-lysine-treated plates at a seeding density of 5 x 10^5 cells/cm^2 in high glucose (4.5 mg/L) Dulbecco's modified Eagle's medium (D-MEM) with 30mM glucose, 30mM KCl, 2mM glutamine, 2.2g/L sodium bicarbonate, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 100 μg/ml streptomycin. After 48 hrs, 20μM cytosine arabinoside was added to the culture medium to inhibit mitotic cell growth. Cells were harvested days 6-8 post seeding.

Primary whole rat brain astrocytes were a generous gift from Dr. R. Armstrong. Briefly, mixed glial cultures were initially seeded from dissected brains of male Sprague-Dawley rats and enriched to virtually pure populations of astrocytes by established procedures [7]. The enriched astrocyte cultures were maintained in high glucose D-MEM supplemented with 1mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.1mM non-essential amino acids, along with above stated concentrations of glutamine, FBS, and penicillin and streptomycin

(Pen/Strep). All primary astrocyte cultures were used between one to two weeks of plating (~ 90% confluence).

All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA), except NBFL cells, which were a generous gift from Dr. A. Symes. Minimum essential media (MEM) was purchased from Life Technologies (Grand Island, NY) supplemented with 1mM Earle's salts. Hep-3B cells were cultured in minimal essential medium (MEM) containing 1mM sodium pyruvate, 1.5 g/L L-glutamine, 10% FBS, 0.1mM non-essential amino acids, and Pen/Strep. U-87 and U-251 cells were cultured in low glucose (1.1 mg/L) D-MEM containing pyruvate, glutamine, FBS, and non-essential amino acids in the presence of Pen/Strep. PC-12 cells were grown in a similar medium with 5% horse serum. SHSY-5Y cells were cultured in MEM and F-12 nutrient mixture (Ham) at a 1:1 (v:v) ratio supplemented with 1.5 g/L sodium bicarbonate, L-glutamine, pyruvate, non-essential amino acids, FBS, and Pen/Strep. All cells were grown in a humidified incubator at 37 °C in a 5% CO_2 atmosphere. Media was routinely replaced 2-3 times weekly. Sub-culturing confluent flasks involved adding trypsin (0.25%) -EDTA (0.03%) solution after removing expired media. Detached cells in a fresh media suspension were transferred to new flasks typically at a subculture ratio of 1:3.

All cultured cells were harvested typically at 80-90% confluence by mechanical scraping in the presence of an ice-cold buffer containing 20mM

HEPES-KOH (pH 7.35), 0.25M sucrose, 100μM EDTA, 0.1 mg/ml phenylmethylsulfonylfluoride (PMSF), and 10 μg/ml each of aprotinin and leupeptin. Cells were pelleted by centrifugation at 1000 x *g* and resuspended in cold HEPES-KOH buffered sucrose in the absence of EDTA and protease inhibitors. Protein concentrations were determined by the Bradford method using protein dye concentrate (Bio-Rad, Hercules, CA) and adjusted to a concentration of 1.25 – 5.0 mg/ml prior to performing Ca^{2+} uptake assays.

Tissue Preparation

Male Sprague-Dawley rats were sacrificed (CO_2 narcosis followed by decapitation); brains were rapidly removed and placed in 10 volumes of ice-cold homogenization buffer (w/v) containing 25mM *N*-2hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid (HEPES) [pH 7.3 with potassium hydroxide (KOH)], 0.25M sucrose, 100mM EDTA, and protease inhibitors. Protease inhibitors were PMSF (100 mg/ml), leupeptin (10 μg/ml), and aprotinin (10 μg/ml). Microsomes were prepared by mincing brain well and then homogenizing with a T-Line laboratory stirrer (Talboys Engineering Corp, Montrose, PA) using a glass-teflon homogenizer at 70% speed with five up and down strokes. Homogenates were centrifuged at 10,000 x *g* with a Sorvall SS-34 rotor in a Beckman centrifuge at 4 °C for 15 min. Supernatant was then centrifuged at 100,000 x *g* with a Sorvall

TH-641 swinging bucket rotor in a Sorvall Ultra Pro 80 ultracentrifuge at 4 °C for 60 min. The pellet was rinsed and re-suspended using a microsomal storage buffer consisting of cold homogenization buffer lacking EDTA and enzyme inhibitors. Protein content was determined by the Bradford method using protein dye concentrate (Bio-Rad, Hercules, CA) and adjusted to a concentration of 1.25 – 5.0 mg/ml prior to storage at –70 °C.

Fresh organ-derived crude cell homogenates were prepared as outlined for microsomes except the initial homogenate was directly centrifuged at 100,000 x *g* and resulting pellet resuspended. For cell cultures in 162 mm^2 flasks, culture media was aspirated at confluence and flasks rinsed with 5 ml of homogenization buffer. Cells were scraped in 5 ml of fresh homogenization buffer, and the resulting suspension placed in a 15 ml conical and centrifuged for 5 min at 3000 x *g*. The pellet was re-suspended in a small volume of microsomal storage buffer, and protein content was adjusted to 2.5 mg/ml.

Fresh frozen sections were obtained by rapidly freezing brain in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). Cryostat sections (18 μm) were thaw mounted at –20 °C onto Fisherbrand Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at –70 °C.

Ca^{2+} Uptake Assay

Accumulation of $^{45}Ca^{2+}$ into microsomes was studied in 20mM HEPES-KOH (pH 7.3) uptake buffer containing 3% (w/v) polyethylene glycol (PEG, avg mol wt 10,000), 5mM sodium azide, and 200μM total $CaCl_2$, with freshly added 5mM 1,4-dithiothreitol (DTT), 5mM phosphocreatine, 2mM ATP, 1mM $MgCl_2$, 20 U/ml creatine phosphokinase, and 0.1 μCi/ml $^{45}Ca^{2+}$. All other additions are as indicated. Free calcium (pCa^{2+}_{free}) levels were adjusted to a desired concentration of 0.25μM with EGTA using an Orion Ca^{2+}-sensitive electrode calibrated with commercially obtained free calcium standards (World Precision Instruments, Sarasota, FL). To initiate $^{45}Ca^{2+}$ uptake, microsomes were added to a final protein concentration of 100 μg/ml in a total reaction volume of 0.25 ml at 37°C. Non-specific uptake was considered as any $^{45}Ca^{2+}$ signal in the presence of 10μM A23187, a calcium ionophore. Microsomal transport of $^{45}Ca^{2+}$, unless otherwise specified, is expressed as specific uptake in which any signal in the presence 10 μM A23187 was subtracted out. Assays routinely proceeded for 60 min and were terminated by rapid filtration through Millipore (Bedford, MA) MAFB filter 96-well microplates. Filters were washed twice with 0.2 ml of ice-cold wash buffer containing 20mM HEPES-KOH (pH 7.3), 3% PEG, 25mM monobasic potassium phosphate, 25mM dibasic potassium phosphate, 5mM sodium azide, 5mM $MgCl_2$, and 2mM EGTA. Alternatively, $^{45}Ca^{2+}$ uptake experiments using chloride as the predominant anion were washed with 100mM KCl instead of

monobasic and dibasic phosphate, while those using oxalate were washed with buffers containing 25mM potassium oxalate. Radioactivity was measured in 0.05 ml of CytoScint liquid scintillation cocktail (ICN Pharmaceuticals, Inc., Costa Mesa, CA) using a Wallac (Gaithersburg, MD) model 1450 microbeta counter.

Histochemical $^{45}Ca^{2+}$ uptake assays were performed in serial frozen sections. The uptake buffer contained 80mM KCl and 10mM potassium oxalate in place of potassium phosphates for better localization. Incubations were performed in polypropylene slide mailing vessels (Evergreen Scientific, Los Angeles, CA) at 37 °C for 30 to 60 min. Slides were transferred to ice-cold wash buffer to terminate the incubation. After washing twice for 2 min, sections were dried under a cool air stream accompanied by vacuum aspiration of excess fluid and then exposed to beta particle sensitive film (Fuji Medical X-Ray Film, Fuji, Stamford, CT) for 24-48 hrs to image the anatomical distribution of accumulated $^{45}Ca^{2+}$. After developing film, brain sections were wiped from the slides with wet absorbent tissue paper and the accumulated radioactivity measured in 3 ml CytoScint scintillation cocktail using the microbeta counter.

Ca^{2+} Leak/Release Assay

Microsomal uptake and release of Ca^{2+} was measured using radiotracer $^{45}Ca^{2+}$ in uptake assays performed in 96-well MAFB microplates as described in a

previous section with modifications. Routine uptake assays were performed for 90 min in the absence and presence of TG (10 nmol/mg protein). At 90 min (which approximates uptake saturation in phosphate-supported buffer), Ca^{2+}-ATPase inhibitors were added and the assay allowed continued. Microsome calcium flux was subsequently monitored by vacuum filtration at different intervals and scintillation counting. Non-specific uptake was considered $^{45}Ca^{2+}$ accumulation in the presence of 10 μM A23187. Calcium leakage was defined as the decrease in specific $^{45}Ca^{2+}$ at various times following the addition of inhibitors compared to saturation at 90 min. Alternatively, $^{45}Ca^{2+}$ uptake/leak assays were performed in 50 ml conicals at high volume (~ 15 ml) while maintaining protein content, [$^{45}Ca^{2+}$], and concentrations of assay reagents similar to small volume assays. For optimal effects of calcium releasing agents, small volume (96-well) assays were utilized by performing $^{45}Ca^{2+}$ uptake while including the releasing agent in the reaction from the beginning of the experiment. Under these conditions, the releasing agent apparently binds to its receptor/calcium release channel and maintains it in an open configuration. The difference in net calcium accumulation in the presence of these agents, therefore, specifically determines the release sensitivity of microsomes to these agents [141].

Phosphorylated Ca^{2+}-ATPase Intermediate Assay

The Ca^{2+}-dependent phosphorylated enzyme intermediate assay was carried out on ice by utilizing [γP^{32}]-ATP (6000 Ci/mmol) from NEN (Boston, MA) as described [70] with modifications. Where applicable, microsomes were pre-incubated on ice with treatments for 5 min in the absence of Ca^{2+}. Microsomes were then suspended at 0.25 mg/ml final protein concentration in 20mM HEPES-KOH (pH 7.0) buffer containing 80mM KCl, 5mM $MgCl_2$, and 110μM $CaCl_2$. Addition of 50nM ATP (300 Ci/mmol) started the reaction. After one min, reaction was quenched by addition of 1 ml ice-cold stop solution containing 12% (w/v) trichloroacetic acid (TCA) and 20mM phosphoric acid. Samples were briefly vortexed and remained on ice for 15 min prior to collection of precipitated proteins. Quenched samples were sedimented in a Beckman microfuge at 14,000 rpm for 5 min. After washing three times in stop solution, the final sediment was dissolved in acidic SDS sample buffer (150mM Tris-HCl, pH 6.8; 10mM EDTA; 30% sucrose; 0.014% bromophenol blue; 2% SDS and 5% mercaptoethanol) and placed in a boiling water bath for 5 min.

Samples were separated for determination of radioactive phosphorous and protein by acidic SDS-PAGE. The stacking gel contained 5% acrylamide with a 40:1 (w/w) ratio of acrylamide:bisacrylamide. The separating gel was comprised of 6% acrylamide with the same 40:1 acrylamide:bisacrylamide ratio.

Reservoir buffer contained a 0.17 MOPS buffer (pH 6.0 with 1.7M Tris base) with 0.1% SDS. Equal amounts of protein were loaded. Gels were run at 100mV for approximately 1.5 hrs. Electro-transfer of single gels at 30mAmps for 1 hr onto 0.2-μm pore nitrocellulose membranes was accomplished in a buffer containing 25mM Tris, 190mM glycine, and 20% methanol. Coomassie Blue staining of gels after blotting and the transfer of molecular weight standards (Sigma) separated in parallel lanes demonstrated efficient protein transfer. Resulting nitrocellulose membranes were subjected to autoradiography.

Measurement of Ca^{2+}-ATPase Activity

Ca^{2+}-ATPase activity was determined after performing $^{45}Ca^{2+}$ uptake assays modified to accommodate colorimetric measurement of liberated inorganic phosphate. Ca^{2+} uptake was performed in a 20mM HEPES-KOH (pH 7.3) buffer containing 80mM KCl, 10mM potassium oxalate, 5mM sodium azide, and concentrations of free Ca^{2+} varied by adding $CaCl_2$ or removing it with chelex 100 resin (BioRad) followed by vacuum filtration. After adjusting the pCa^{2+}, 3% PEG was added along with fresh additions of 5mM DTT, 1mM $MgCl_2$, 0.1 μCi/ml $^{45}Ca^{2+}$, and brain microsomes (100 μg/ml final protein concentration). Reactions were started by the addition of 2mM ATP in a final volume of 250 μl, and continued at 37 °C for 30 min. Vacuum filtration of the 96-well MAFB microplate

into a polystyrene 96-well culture microplate halted the uptake assay. Ca^{2+}-dependent ATPase activity was subsequently determined by measuring liberated phosphate in the filtrate using a described method [150] with minor modifications. A solution containing 2% ammonium molybdate in 1.8M H_2SO_4 and 5% (v/v) of W-1 polyoxyethylene ether was added to the filtrate (100 µl per well). A microwell plate reader was used to immediately measure the formation of colored phospho-molybdate complex in the filtrate at 410 nm. Ca^{2+}-activated ATP hydrolysis was expressed as relative calcium-dependent increase in absorbance.

Statistical Analysis:

All data collected was parametric in nature, and consisted of treating the same sample tissue preparations with different treatments. The primary statistical test used to analyze experimental data for significant difference was two-tailed Student's *t* test for paired samples. Typically, three independent experiments were performed prior to data analysis. Although the majority of experiments utilized pooled rat brain from 8 male Sprague-Dawley rats and an argument could be made for using N=8 multiplied by the number of replicates, an N=3 to represent mean values from each independent performed experiment was used for more conservative analyses. Statistical significance was considered to be $p < 0.05$.

RESULTS

Thapsigargin distinguishes two Ca^{2+} uptake pools in rat brain

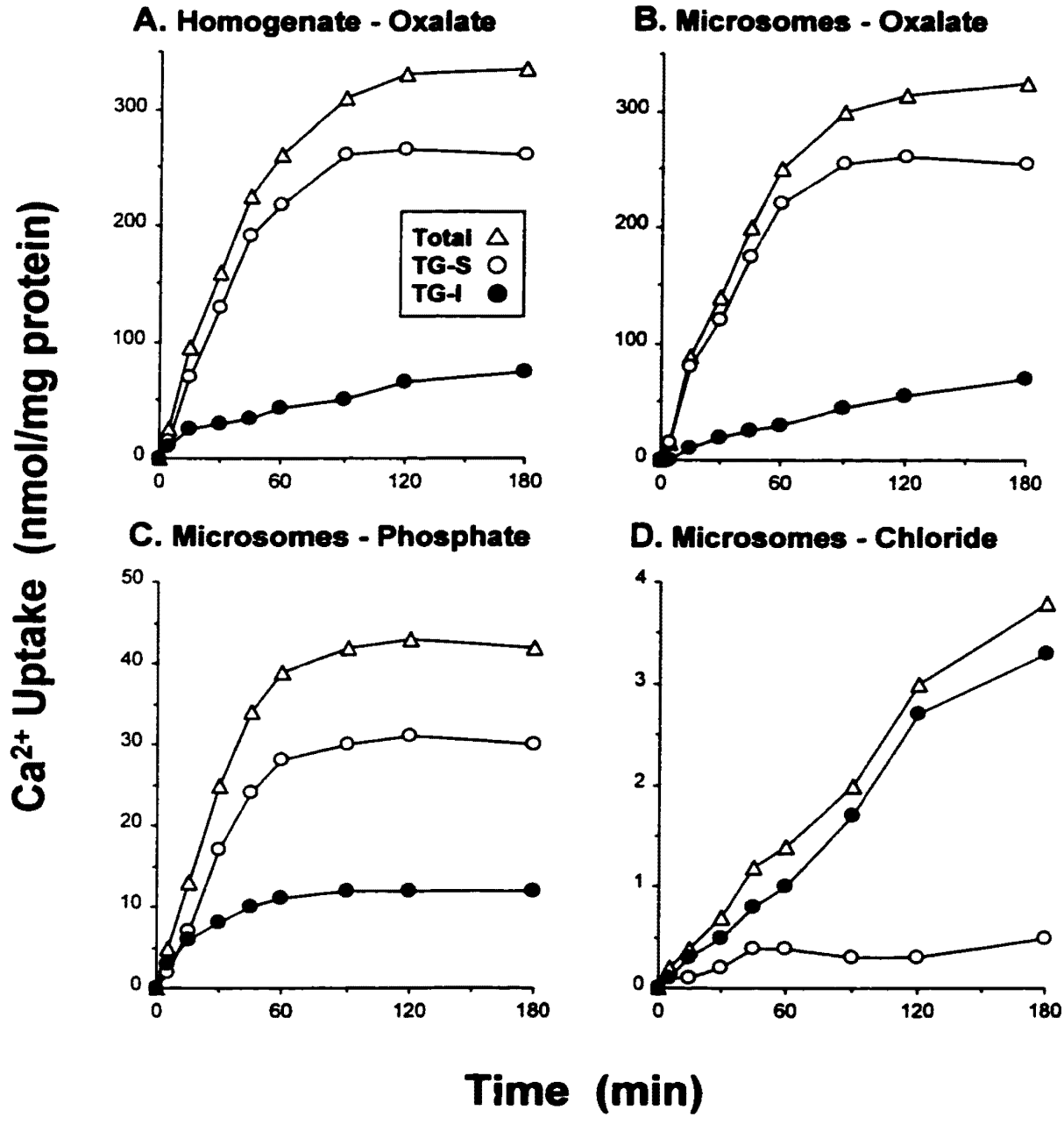
To investigate the properties of rat brain Ca^{2+} sequestering compartments, we monitored Ca^{2+} transport into homogenates and microsomes as described in METHODS. Vesicles derived from the sarco-endoplasmic reticulum (SR-ER) can accumulate Ca^{2+} using the energy derived from ATP hydrolysis [51, 52, 134]. To study the TG-I Ca^{2+} pool specifically, we utilized Mg^{2+} and ATP-dependent $^{45}Ca^{2+}$ uptake in rat brain preparations in the presence of TG. The Ca^{2+} uptake buffer we utilized contains an ATP-regenerating system and sodium azide, an inhibitor of the F_1F_0 ATPase, which effectively excluded mitochondria from contributing to net ATP-dependent Ca^{2+} sequestration [139, 140]. Crude homogenate preparations and microsomes potentially contain revesicularized plasma membranes along with its resident Ca^{2+} ATPase, the PMCA. The PMCA is not sensitive to TG inhibition. Thus, it is possible that revesicularized plasma membrane could contribute to the TG-I Ca^{2+} uptake. Digitonin, a saponin detergent, can selectively permeabilize the cholesterol-rich plasma membrane at low concentrations [140]. A 10μM concentration of digitonin maximally permeabilizes the plasma membrane and this concentration was found to have no effect on Ca^{2+} accumulation in either brain homogenates(data not shown) or microsomes (Figure 10). To safeguard

against the variable contribution of plasma membrane vesicles to Ca^{2+} accumulation in different preparations, 10μM digitonin was routinely included in all assays. To distinguish Ca^{2+} accumulation into membranous compartments from non-specific Ca^{2+} binding, we utilized the Ca^{2+}-specific ionophore, A23187. This compound prevents the ability of membranous compartments to concentrate Ca^{2+}, and potently inhibited Ca^{2+} accumulation in our assays with an IC_{50} of 3nM and maximal inhibition at 100nM. In all assays, any signal measured in the presence of 10μM A23187 was thus subtracted out to monitor only specific Ca^{2+} accumulation.

Ca^{2+} accumulation in rat brain crude homogenates (Figure 2A) and microsomes (Figure 2B) performed in a buffer containing oxalate as the sole co-transported anion display progressive uptake activity over a three hour time course. By utilizing 100nM TG (1 nmol/mg), a dose which effectively inhibits SERCA activity [110, 116], the total Ca^{2+} accumulation can be resolved into two distinct activities referred to as the TG-sensitive (TG-S) and the TG-insensitive (TG-I) components. The uptake activity in isolated microsomes is qualitatively and quantitatively similar to that of crude homogenates, indicating that the microsomal preparation contains the majority of ER-like Ca^{2+} accumulating compartments. By subtracting the TG-I activity from total Ca^{2+} accumulation, the TG-S component is selectively displayed. Ca^{2+} accumulation into the TG-S and

Figure 2. Thapsigargin-resistant Ca^{2+} accumulation in rat brain homogenates and microsomes. Energy-dependent $^{45}Ca^{2+}$ uptake was measured over 3 hrs in rat whole brain crude homogenates (A) and microsomes (B-D) utilizing buffers in which the predominant anion was either oxalate (top two panels), phosphate (lower left), or chloride (lower right). Calcium uptake was determined in the absence and presence of thapsigargin (100nM or 1nmolmg protein) as described in Materials and Methods. **A.** In the absence of thapsigargin, rat brain homogenates display robust Ca^{2+} accumulation in oxalate-based buffers (total, open triangles) which approaches saturation at approximately 2 hrs. Although Ca^{2+} uptake is markedly diminished in the presence of thapsigargin, a distinct thapsigargin-insensitive component (TG-I, dark circles) continues to increase linearly representing ~ 30% of total accumulation at 3 hrs. By subtracting the TG-I component from the total, a thapsigargin sensitive component (TG-S, open circles) can be distinguished from the TG-I. **B.** Oxalate-supported rat brain microsomal Ca^{2+} accumulation accounts for most of the uptake seen in homogenates and is also composed of distinct TG-I and TG-S components. **C.** Phosphate-supported Ca^{2+} uptake in brain microsomes is less robust than with oxalate and shows much earlier saturation of both the TG-S and TG-I components with the TG-I representing a greater proportion of the overall $Ca2+$ accumulation. **D.** In chloride-based buffers microsomal Ca^{2+} uptake is markedly reduced and primarily represents the TG-I component. In all Ca^{2+} uptake experiments, non-specific signal, as measured in the presence of 10mM A23187, was subtracted out. Each data point represents the mean of assessments performed in triplicate and the graphs shown are representative of two experiments which varied less than 5%.

FIGURE 2


A. Homogenate - Oxalate
B. Microsomes - Oxalate
C. Microsomes - Phosphate
D. Microsomes - Chloride
Total
TG-S
TG-I
Ca2+ Uptake (nmol/mg protein)
Time (min)

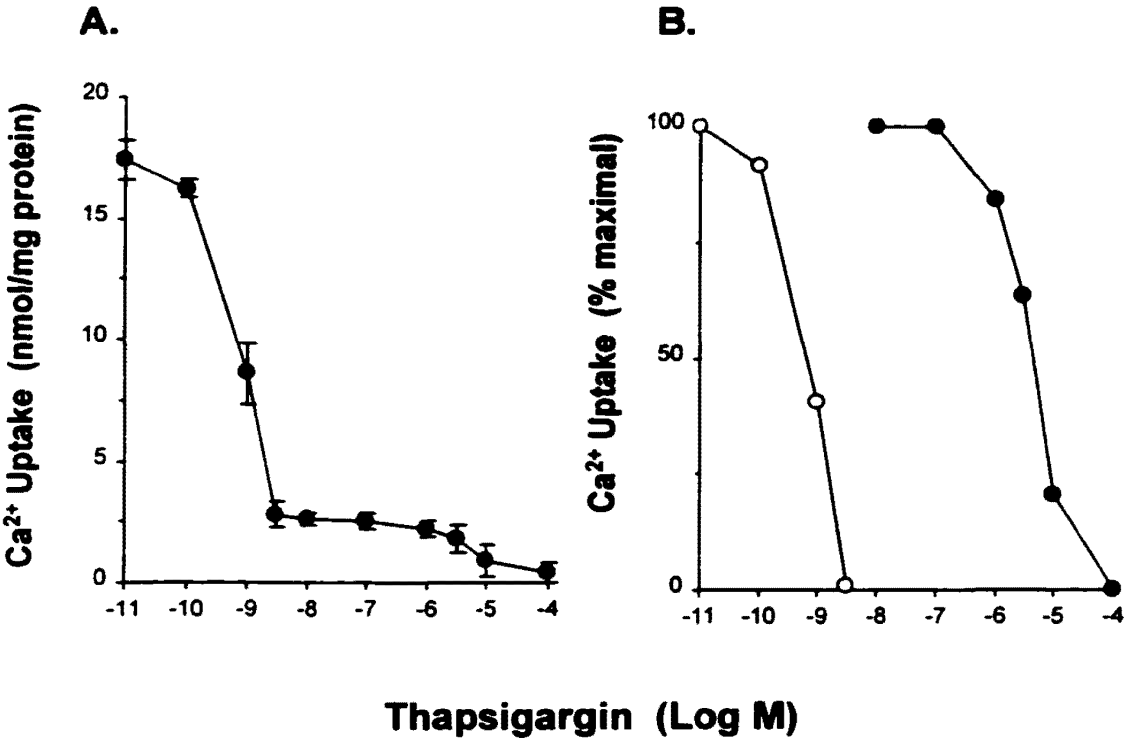
TG-I components can be distinguished by their kinetics. TG-S Ca^{2+} uptake in oxalate-supported assay buffer appears to saturate by 2 to 3 hrs incubation while TG-I Ca^{2+} uptake increases linearly over 3 hrs of incubation. The relative contribution of TG-S and TG-I to the total Ca^{2+} accumulation in our experiments is highly dependent on the predominant anion present in the buffer. In buffers which contain oxalate as the predominant anion the TG-S and TG-I components represent 86.4 and 13.6% of the total Ca^{2+} accumulation at 1 hr, respectively. With phosphate-based buffers the TG-S and TG-I components appeared to saturate by ~ 1 hr. At this time point, the TG-S and TG-I components represent 60.7 and 39.3% of the total Ca^{2+} accumulation (Figure 2C) with this ratio being variable with time prior to saturation. Compared with oxalate, the phosphate-based buffer has lower overall Ca^{2+} accumulation but a greater proportion of the TG-I component. In chloride-based buffers, overall Ca^{2+} accumulation is further reduced, however, a minimal sensitivity to TG-inhibition is observed (Figure 2D). Thus, the detection of the TG-I component of overall Ca^{2+} accumulation is highly dependent on assay conditions. Subsequent comparisons of the TG-S and TG-I compartments were therefore performed in phosphate-containing buffers at 60 min incubation times. The kinetic studies described above were performed in glass test tubes and harvested over GF/B filters which were pre-soaked 0.03% polyethyleneimine (PEI). PEI treatment imparts a positive charge to the glass fiber filters, which increases protein capture by the filters while reducing non-specific $^{45}Ca^{2+}$ binding to the filters. In subsequent studies uptake reactions were carried out

in Millipore 96-well MAFB filter microplates, which could be filtered using a vacuum manifold and counted in a 96-well plate radiation counter. PEI could not be used with these plates. Results obtained using this methodology were qualitatively similar to those presented above, however, lower total amount of Ca^{2+} accumulation per mg protein was seen.

Thapsigargin dose-dependently inhibited Ca^{2+} uptake in phosphate-based buffers in a biphasic manner (Figure 3A) similar to our previous findings in studies with brain microsomes using buffers which contained a combination of oxalate and chloride [140, 141]. Over the dose range of 10pM - ~ 1nM, TG produced ~ 80% inhibition of the overall Ca^{2+} accumulation and this component represents the TG-S described above. The potent inhibitory effect of TG is consistent with its action on all known SERCAs [83]. The ~ 20% of Ca^{2+} accumulation which remains (TG-I) is inhibited by TG only at high micromolar concentrations. This is consistent with several other reports examining TG-I Ca^{2+} accumulation in cultured cell lines (see [143] for an example). In Figure 3B the amount of Ca^{2+} accumulation remaining in the presence of 10nM TG was subtracted from the total to resolve the TG-S and TG-I components. Ca^{2+} uptake expressed as percent inhibition of the two distinct pools clearly shows that TG-S component can be distinguished from the TG-I component by its > 1,000-fold higher sensitivity to TG.

Figure 3. Thapsigargin (TG) sensitivity distinguishes two distinct components of brain microsomal Ca^{2+} accumulation. **A.** Phosphate-supported $^{45}Ca^{2+}$ uptake was monitored in whole rat brain microsomes for 60 min in the presence of increasing concentrations of TG. A biphasic inhibition of Ca^{2+} uptake is produced by TG with a distinct component of Ca^{2+} uptake being sensitive to TG over a range of 10pM – 10nM. A second inhibitory action of TG on the remaining Ca^{2+} uptake activity is observed over the range of TG from 1mM – 100mM. The two distinct calcium accumulating activities distinguished by high and low sensitivities to TG inhibition are referred to as the TG-S and TG-I components, respectively. The data represent means +/- SE of three independent experiments performed in triplicate. **B.** The TG-S and TG-I components are displayed separately with their respective sensitivities to TG depicted as percent inhibition. For this representation, the TG-S component (open circles) was determined by subtracting Ca^{2+} accumulation in the presence of 10nM TG from the total. The remaining activity represents the TG-I component (closed circles).

FIGURE 3


A.
B.
Ca2+ Uptake (nmol/mg protein)
Ca2+ Uptake (% maximal)
20
15
10
5
0
100
50
0
-11
-10
-9
-8
-7
-6
-5
-4
Thapsigargin (Log M)

To determine whether the TG-I Ca^{2+} uptake in brain microsomes reflects the activity of a Mg^{2+}-ATPase, we studied the dependence of this activity on ATP and Mg^{2+}. Figure 4 shows that Ca^{2+} accumulation by brain microsomes into both the TG-S and TG-I components has an absolute requirement for ATP and Mg^{2+}. In these studies, the two components were distinguished by the presence of 100nM TG. Vanadate, a well known inhibitor of P-type ATPases, inhibits Ca^{2+} uptake in the absence and presence of TG suggesting that TG-I Ca^{2+} uptake may also be mediated by a P-type ATPase.

TG-S and TG-I Ca^{2+} uptake activities display similar dependencies for ATP, Mg^{2+}, and Ca^{2+}

The ATP, Mg^{2+}, and Ca^{2+}-dependencies of Ca^{2+} accumulation in brain microsomes were studied in the absence and presence of TG (1 nmol/mg protein). The data shown in Figure 5A, B, and C demonstrated that the total and TG-I Ca^{2+} uptake components were similarly dependent on ATP, Mg^{2+}, and Ca^{2+}, respectively. Increasing concentrations of these reagents results in increased uptake rising to a peak activity. In all cases, the effective concentrations of these agents producing peak activity is approximately the same for both TG-S and TG-I components. Maximal uptake occurs at about 1mM for ATP; 2mM for Mg^{2+}; and 5μM for Ca^{2+} (Figure 4 and Table 2). The bell-shaped dependencies of uptake activity for the TG-S and TG-I Ca^{2+} uptake

components depicted has been described for enzymatic activity of Ca^{2+}-ATPases [48].

Figure 4. TG-I Ca^{2+} as uptake requires ATP and Mg^{2+}, and is inhibited by vanadate. To determine the relative dependencies of the TG-S and the TG-I on ATP and Ca^{2+}, uptake assays were performed as in Figure 2, but with or without 100nM TG added to the buffer. While the TG-S (open bars) component was larger in magnitude than the TG-I component (filled bars), both pools showed a similar dependency on ATP and Mg^{2+} as well as inhibition by vanadate when expressed in terms of percentage, as in Figure 3.

FIGURE 4

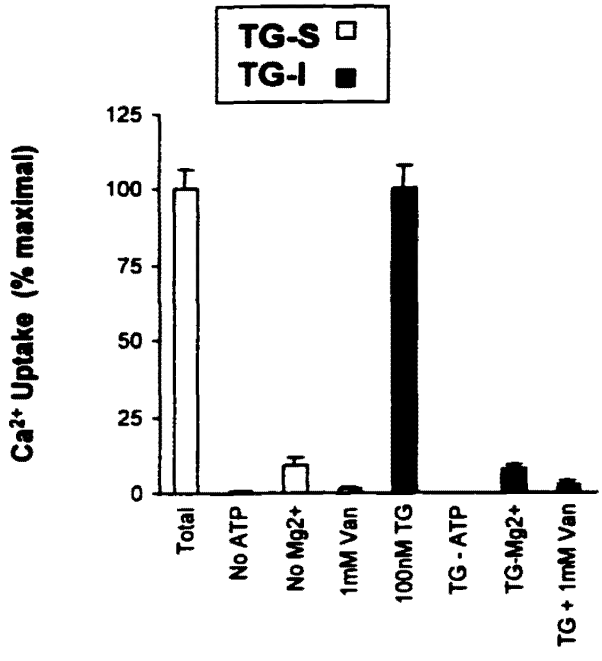
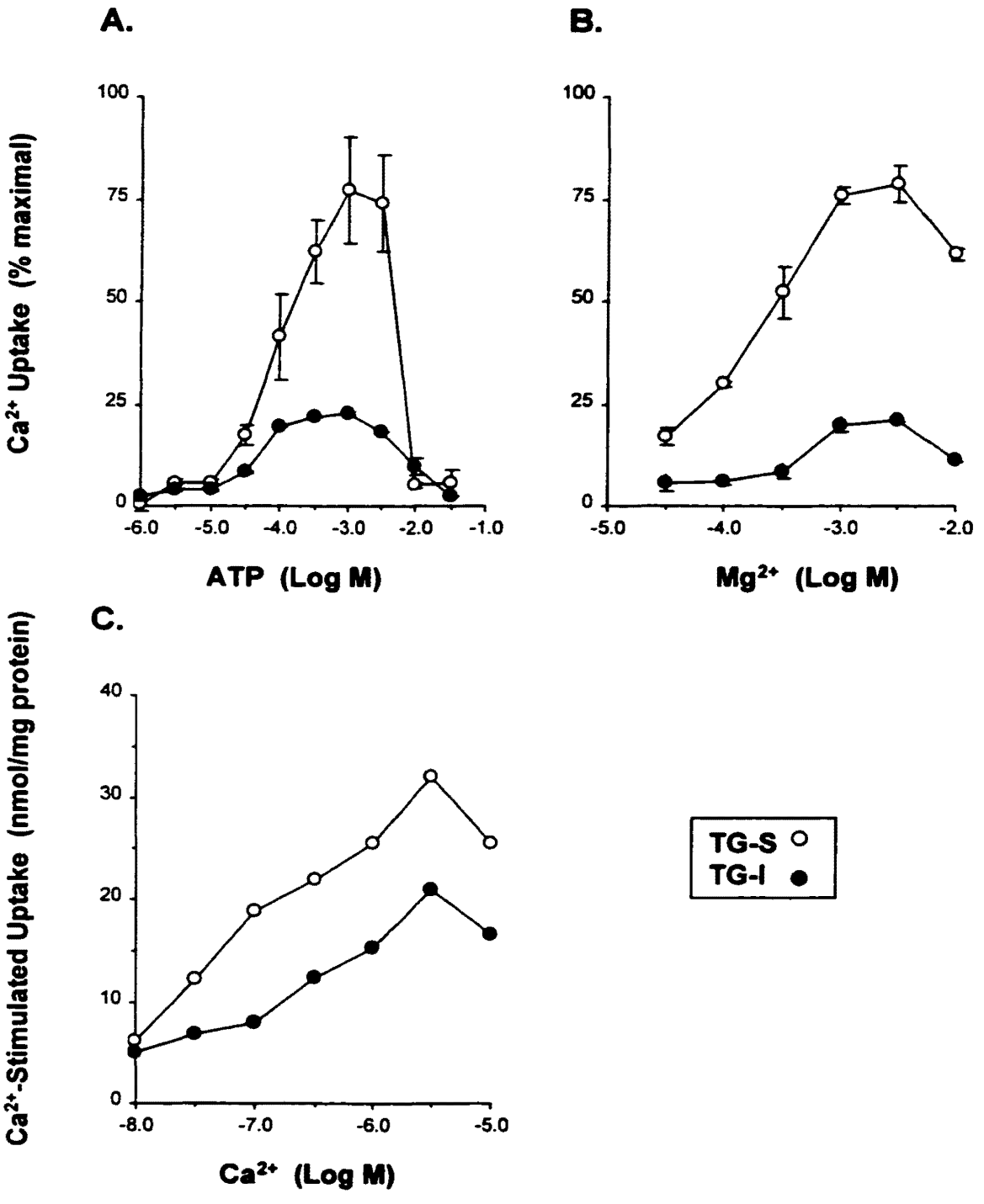

TG-S
TG-I
Ca2+ Uptake (% maximal)
125
100
75
50
25
0
Total
No ATP
No Mg2+
1mM Van
100nM TG
TG - ATP
TG-Mg2+
TG + 1mM Van

Figure 5. Optimal ATP, Mg^{2+}, and Ca^{2+}concentrations are similar for TG-I and TG-S components. Uptake assays were performed as in Figure 3, but over a varying range of ATP and Mg^{2+} concentrations (**5A** and **B**, respectively). Both agents stimulated TG-S (open circles) and TG-I (closed circles) Ca^{2+} uptake up to a maximum followed by a decline in uptake at higher concentrations. Values represent means +/- SE from three separate experiments, with maximal Ca^{2+} uptake for ATP and Mg^{2+} 23.6 and 24.6 nmol/mg protein, respectively. **C.** To monitor the influence of free Ca^{2+} levels on total and TG-I Ca^{2+} accumulation, buffers containing identical amounts of total Ca^{2+} were titrated to varying free Ca^{2+} levels using EGTA and Ca^{2+}-specific electrode, as described in Methods. Ca^{2+} also activated TG-S and TG-I Ca^{2+} uptake to a maximum at 5mM. Further increase in free Ca^{2+} produced relative inhibition for both Ca^{2+} accumulating activities.

FIGURE 5


A.
B.
C.
100
75
50
25
0
-6.0
-5.0
-4.0
-3.0
-2.0
-1.0
Ca2+ Uptake (% maximal)
ATP (Log M)
-5.0
-4.0
-3.0
-2.0
Mg2+ (Log M)
40
30
20
10
0
-8.0
-7.0
-6.0
-5.0
Ca2+-Stimulated Uptake (nmol/mg protein)
Ca2+ (Log M)
TG-S
TG-I

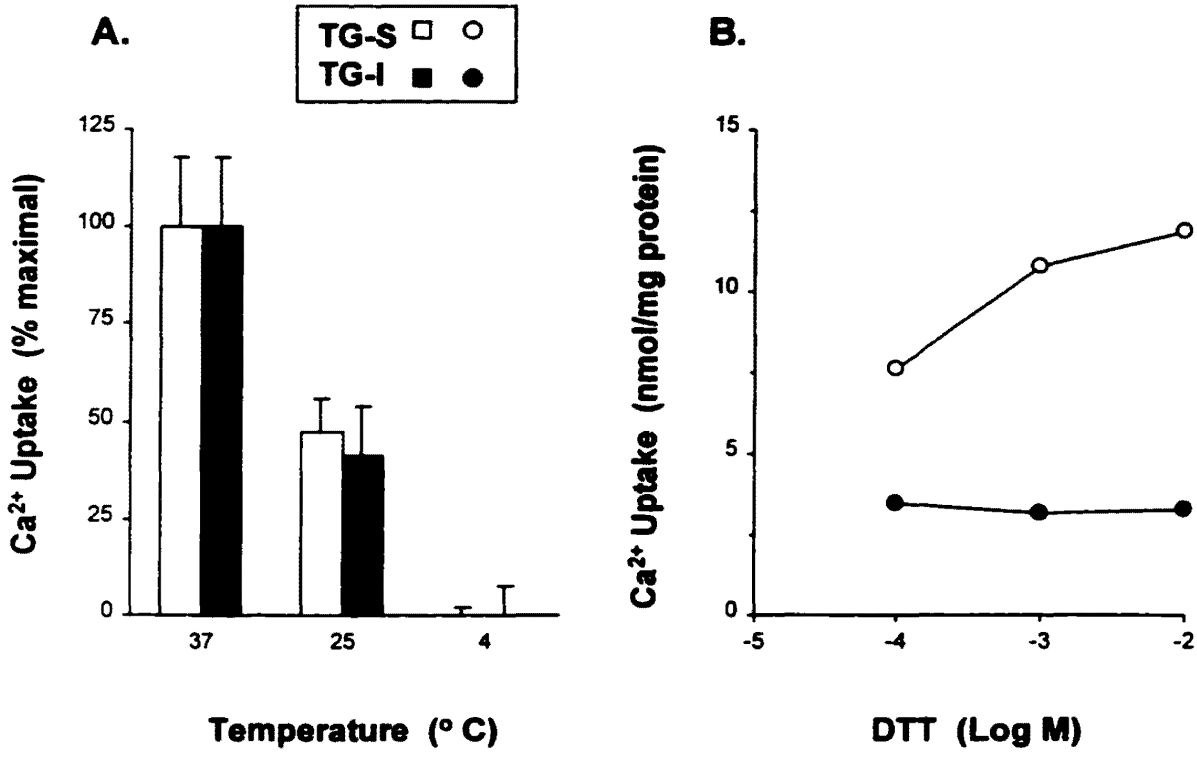
TG-S and TG-I Ca^{2+} uptake mechanisms display similar temperature dependency, but unique sensitivity to redox modification

The sensitivity of TG-S and TG-I Ca^{2+} uptake activities was determined for temperature and dithiothreitol (DTT), as shown in Figure 6A and B, respectively. Both components were similarly sensitive to temperature, with maximum uptake occurring at 37 °C. Decreasing temperature to room temperature resulted in ~ 50% decrease in TG-S and TG-I Ca^{2+} uptake. A further decrease to 4 °C virtually eliminated Ca^{2+} accumulation in either compartment.

The TG-S and TG-I Ca^{2+} uptake activities were found to be uniquely sensitive to DTT, a reagent which perserves protein function by protecting sulfhydryl groups (Figure 6B). The structure and function of many proteins are dependent on the maintenance of sulfhydryl groups (-SH), and SERCA activity is known to be highly sensitive to sulfhydryl group modification and temperature changes [28]. TG-S Ca^{2+} uptake is stimulated by DTT in a dose-dependent manner, displaying a 50% increase observed at a concentration of 10mM as compared to 100μM. In contrast, TG-I Ca^{2+} uptake was insensitive to the presence of DTT over the concentrations used.

Figure 6. TG-I and TG-S Ca^{2+} accumulation have similar temperature dependencies, but unique sensitivity to DTT. Ca^{2+} uptake into TG-S and TG-I components was determined as described in phosphate-supported buffer at different temperatures (**A**) and with different added amounts of DTT (**B**). Both components of Ca^{2+} uptake are similarly temperature dependent while only the TG-S appears to be influenced by DTT. Results are representative of means observed in at least two experiments performed in triplicate. Mean +/- SEM values (n=3) are shown for the temperature sensitivities studies.

FIGURE 6


A.
TG-S
TG-I
125
100
75
50
25
0
Ca2+ Uptake (% maximal)
37
25
4
Temperature (° C)
B.
15
10
5
0
Ca2+ Uptake (nmol/mg protein)
-5
-4
-3
-2
DTT (Log M)

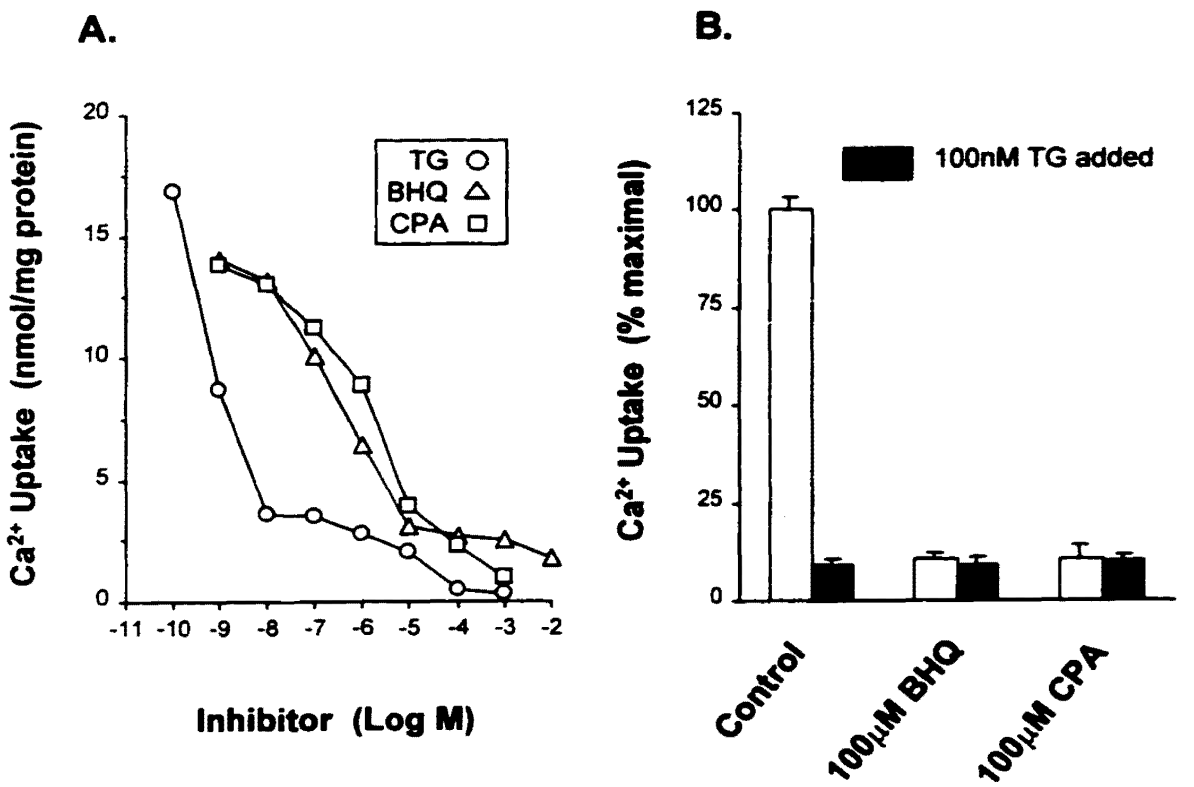
TG-S and TG-I Ca^{2+} uptake are distinguished by sensitivities to other ion-motive ATPases inhibitors

In addition to TG, all known SERCA isoforms are also inhibited by BHQ and CPA (Figure 7A and B). Both of these agents demonstrate dose-dependent inhibition of brain microsome Ca^{2+} uptake with the suggestion of a biphasic pattern similar to that seen with TG (Figure 7A). BHQ and CPA were less potent than TG in inhibiting Ca^{2+} uptake, having IC_{50}'s of 500nM and 5μM, respectively. Similar to the effects seen with TG, the effect of BHQ shown in Figure 7A plateaus between 10 – 100μM. The plateau identified by TG doses between 10nM – 1μM appears equivalent to that demonstrated by BHQ. CPA also appears to level off at the same plateau, although the effect is not as obvious. BHQ and CPA (100μM, 1μmol/mg protein) are not additive with TG (1 nmol/mg protein) (Figure 7B). BHQ and CPA possess chemically distinct structures and presumably inhibit SERCA activity differently compared to TG. This finding further indicates that the TG-S and TG-I Ca^{2+} uptake are mediated by distinct mechanisms.

Ion accumulating mechanisms are ubiquitously expressed in cells, and Ca^{2+} is known to accumulate into many different organelles, such as mitochondria and secretory vesicles. These organelles are thought to accumulate Ca^{2+} through the use of ion exchange mechanisms secondary to the concentration of a different cation within their lumens. Table 2 summarizes

Figure 7. Other SERCA inhibitors also distinguish between the TG-S and TG-I components and are not additive with thapsigargin. Rat brain Ca^{2+} accumulation is inhibited in a biphasic manner by the SERCA inhibitors, BHQ and CPA (**A**). The effects of these agents are non-additive with that of TG (**B**). Results are representative of means +/- SE observed in at least three separate experiments, and indicate that the mechanism responsible for TG-I Ca^{2+} uptake is not mediated by known SERCAs.

FIGURE 7


A.
B.
TG
BHQ
CPA
Ca2+ Uptake (nmol/mg protein)
Inhibitor (Log M)
100nM TG added
Ca2+ Uptake (% maximal)
Control
100μM BHQ
100μM CPA

the effects of several different ATPase inhibitors and ionophores on TG-I Ca^{2+} uptake. As shown above (Figure 4), TG-I Ca^{2+} uptake activity is sensitive to vanadate inhibition, which suggests the involvement of a P-type ATPase. Another recently described PMCA pump inhibitor, eosin Y, appears to equally inhibit TG-S and TG-I Ca^{2+} uptake (IC_{50} ~ 5μM). Other specific ATPase inhibitors are ineffective at inhibiting TG-I Ca^{2+} uptake. Omeprazole and ouabain are specific P-type pump inhibitors for the gastric K^+/H^+-pump and the Na^+/K^+-ATPase, respectively. The lack of effect of these two inhibitors on TG-I activity argues against the involvement of their target ATPases in filling this Ca^{2+} pool. Oligomycin, an ATPase-synthase inhibitor, also has no effect on TG-I uptake, suggesting that mitochondrial uptake is not responsible for TG-I activity. Furthermore, the ATP synthase inhibitor sodium azide was routinely present in the assay buffer of Ca^{2+} uptake experiments, and the free Ca^{2+} was maintained at 250nM, well below the concentration required to stimulate mitochondrial uptake. Bafilomycin, a specific inhibitor of V-type H^+-ATPases which acidify secretory vesicles, is also ineffective at inhibiting TG-I Ca^{2+} uptake. FCCP, a potent protonophore which collapses proton gradients had no effect on TG-I Ca^{2+} accumulation. Monensin, a H^+/Na^+ ionophore, also was unable to prevent loading into the TG-I Ca^{2+} store. In fact, none of the ionophores studied (other than A23187) had any effect on TG-I Ca^{2+} uptake, which strongly argues against an ion exchange process involving H^+, Na^+, or K^+ ions. Collectively, this data suggests that TG-I Ca^{2+} accumulation is not

Table 2. TG-I Ca^{2+} uptake is sensitive to SERCA and general P-type inhibitors, and does not appear to be the result of cation exchange mechanisms. Brain microsomal Ca^{2+} accumulation was performed at optimal ATP and Ca^{2+} concentrations and was measured in the presence of 100nM TG and high doses of known ATPase inhibitors or ionophores. Data are representative of results from at least three independent experiments performed in triplicate. TG-I Ca^{2+} uptake was inhibited by very high concentrations of known SERCA inhibitors (under the heading, "Known SERCA inhibitors"), as well as by the general ATPase inhibitors vanadate and eosin. Concentrations of other specific cation pump inhibitors up to ten-fold above their known effective dose had no effect on TG-I. The inhibitors evaluated were ouabain, omeprazole, oligomycin, and bafilomycin. The target ATPases and the effective dose of these inhibitors is listed in the table. Similarly, ionophores specific for Ca^{2+} and several other cations (Na^+/H^+/ and K^+) were monitored for their effects on TG-I Ca^{2+} uptake, and the results listed under "Ionophores" in the table. Only the Ca^{2+}-specific ionophore, A23187, had any effect on the TG-I compartment. Results demonstrate that the TG-I mechanism is not mediated by classical SERCA activity, and the Ca^{2+} sequestration into the TG-I compartments is not due to secondary exchange of Ca^{2+} for another cation.

Table 2. Pharmacology of TG-I Ca^{2+} Uptake in Brain Microsomes

Reagent	IC_{50} or EC_{50}
Calcium	5μM (peak accumulation)
ATP	1-2mM (peak accumulation)
Magnesium	1-3mM (peak accumulation)
Known SERCA Inhibitors (SERCA IC_{50}):	
Thapsigargin (1nM)	3μM
Cyclopiazonic acid (5μM)	1mM
BHQ (500nM)	> 100μM
Vanadate (100μM)	100μM
Other ATPase Inhibitors (ATPase inhibited: effective inhibitory doses):	
Eosin Y (PMCA; 40μM)	1-10μM
Omeprazole (K^+/H^+ Pump; 10μM)	> 100μM
Oligomycin (F_1F_0ATPase; 100μM)	> 1mM
Ouabain (Na^+/K^+ ATPase; 10μM)	> 100μM
Bafilomycin (vesicular H^+ ATPase; 100nM-1μM)	> 100nM
Ionophores (Specific ion):	
A23187 (Ca^{2+})	3nM
Nigericin (Na^+/H^+; 1μM)	> 10μM
Gramicidin ($H^+/K^+/Na^+$; 1μM)	> 10μM
Monensin ($H^+/K^+/Na^+$; 1μM)	> 10μM
FCCP (H^+; 1μM)	> 10μM

mediated by ion exchange mechanisms, and does not involve significant contributions from mitochondria or acidified compartments such as secretory vesicles, lysosomes, and endosomes.

TG-I Ca^{2+} uptake mechanism is associated with ATPase activity and phospho-enzyme intermediate formation

TG-I Ca^{2+} uptake was studied for the association of Ca^{2+} transport and ATP hydrolysis. Ca^{2+} uptake buffers containing a concentration curve of free Ca^{2+} were made by chelex resin (immobilized EDTA) titration. As described in METHODS, a routine uptake assay was performed on brain microsomes in filter microplates for 30 min, after which vacuum-filtration was performed. A clear plastic 96-well microplate was strategically placed in the filtration path to collect the filtrate. An aluminum molybdate solution added to the filtrate reacted with liberated phosphate forming a colored complex which was measured spectrophotometrically at 410 nm. This method directly couples Ca^{2+} uptake activity and the measurement of ATP hydrolysis. Since we sought to measure ATP activity by monitoring the production of liberated phosphate, an oxalate-supported buffer was utilized instead of the standard phosphate-based buffers routinely used otherwise. Figure 8A demonstrates the total and TG-I Ca^{2+} uptake components are similarly stimulated by varying concentrations of free Ca^{2+}. Absorbance activity at 3nM free Ca^{2+} was assumed to represent the activity of Ca^{2+}-independent, Mg^{2+}-dependent ATPases ubiquitous in most cellular and subcellular preparations, including microsomes. Increasing

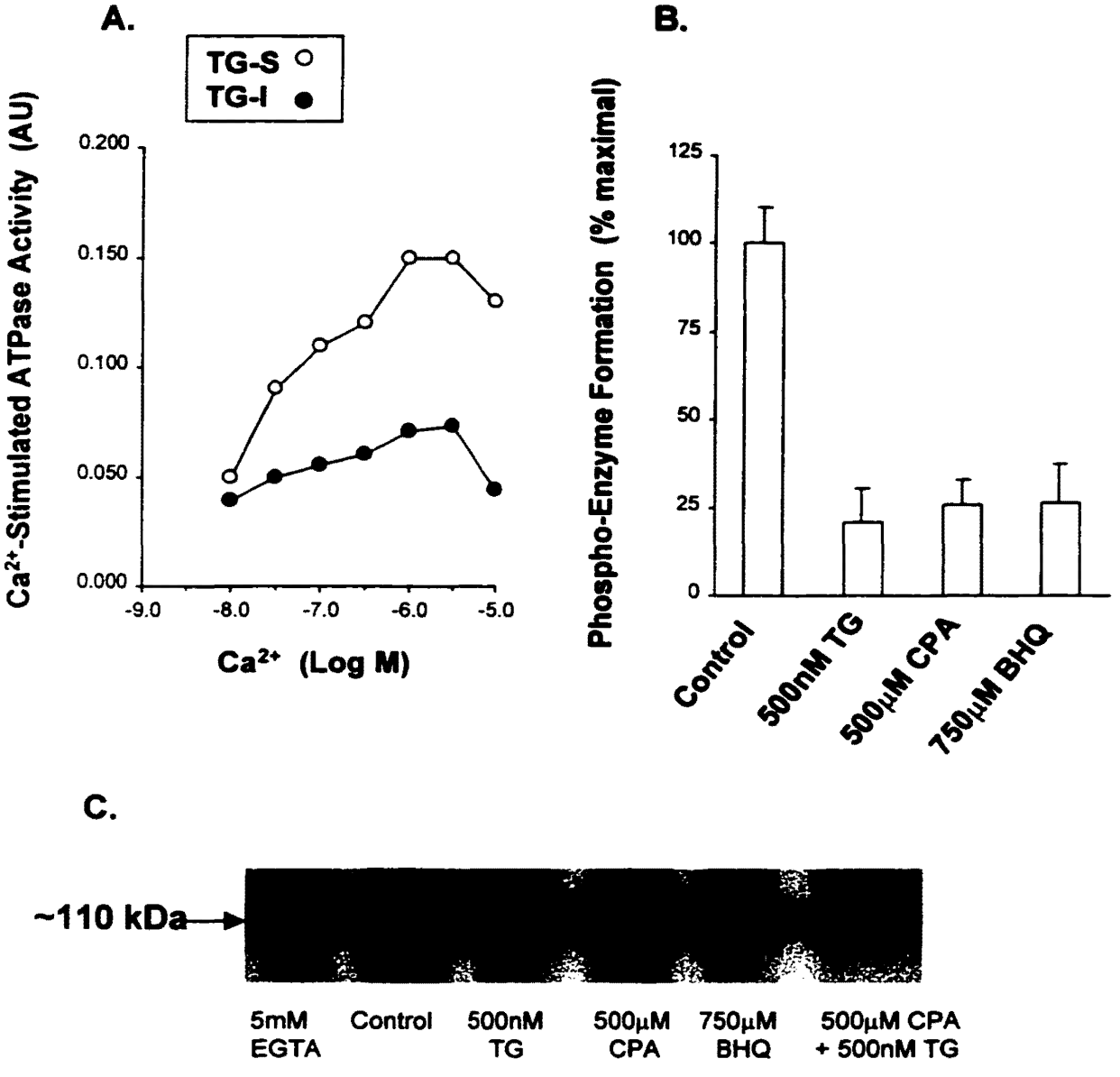
concentrations of free Ca^{2+} stimulate the ATP hydrolytic activity producing a bell-shaped curve to a peak at approximately 5μM for total and TG-I activities (Figure 8A). Increasing free Ca^{2+} above 5μM apparently inhibited pump activity, an observation similar to that reported in other brain microsomal preparations [48, 116].

To further explore the involvement of a P-type ATPase in mediating the TG-I Ca^{2+} accumulation, we determined whether a Ca^{2+}-stimulated [^{32}P] phospho-enzyme intermediate that was insensitive to SERCA inhibitors could be detected in brain microsomes. SERCAs are P-type ATPases which form a covalent phosphorylated enzyme intermediate as part of their Ca^{2+} transport cycle. Although this intermediate normally occurs at a rapid rate, the phospho-enzyme intermediate can be trapped at 4 °C and precipitated with trichloroacetic acid. Brain microsomes form a [^{32}P] phospho-enzyme intermediate in a Ca^{2+}-stimulated manner. While the SERCA inhibitors TG (500nM), CPA (500μM), and BHQ (750μM) inhibit about 75% of total phospho-enzyme formation, about 25% of the phospho-enzyme activity is insensitive to these inhibitors but is sensitive to inhibition by EGTA (5mM) (Figure 8B). Gel electrophoretic analysis of Ca^{2+}-stimulated [^{32}P] phospho-enzyme activity shows a diffuse band at approximately 110 kDa, consistent with the molecular weight of SERCAs (Figure 8C). While EGTA abolishes [^{32}P] incorporation into this region, the SERCA inhibitors are only partially effective. These results suggest

that a P-type Ca^{2+} pump similar to SERCA, but differing in inhibitor sensitivity, may be involved in mediating the TG-I Ca^{2+} accumulation.

Figure 8. TG distinguishes two distinct calcium stimulated ATPase and phospho-enzyme intermediate activities. ATPase assays were performed as described in Materials and Methods. **A.** TG-S and TG-I calcium-activated ATPase activities display a bell-shaped response to varying free calcium levels comparable to that in calcium uptake assays. Values are representative of mean absorbance units (AU) at 410 nm. The experiment was carried out twice with 6 replicates determinations for each data point. **B.** Rat brain microsomes covalently incorporated radioactivity from [γ32P]-ATP at 4oC in a calcium-dependent manner. About 25% of total Ca^{2+}-induced phospho-enzyme formation was not sensitive to TG, BHQ, CPA, or combinations thereof (data not shown). **C.** Acidic gel electrophoresis of phospho-enzyme intermediate reactions revealed a radiolabeled protein band with a molecular weight of about 110 kDa, which corresponds to the mw of known SERCA. SERCA inhibitors alone or in combination only partially inhibited this signal. No labeling was seen in the presence of the Ca^{2+}-specific chelator, EGTA (5mM). These results suggest that a novel Ca^{2+}-stimulated ATPase with an associated phospho-enzyme formation and mw similar to that of known SERCAs mediates uptake into the TG-I compartment.

FIGURE 8


A.
TG-S
TG-I
0.200
0.150
0.100
0.050
0.000
-9.0
-8.0
-7.0
-6.0
-5.0
Ca2+-Stimulated ATPase Activity (AU)
Ca2+ (Log M)
B.
125
100
75
50
25
0
Phospho-Enzyme Formation (% maximal)
Control
500nM TG
500µM CPA
750µM BHQ
C.
~110 kDa
5mM EGTA
Control
500nM TG
500µM CPA
750µM BHQ
500µM CPA + 500nM TG

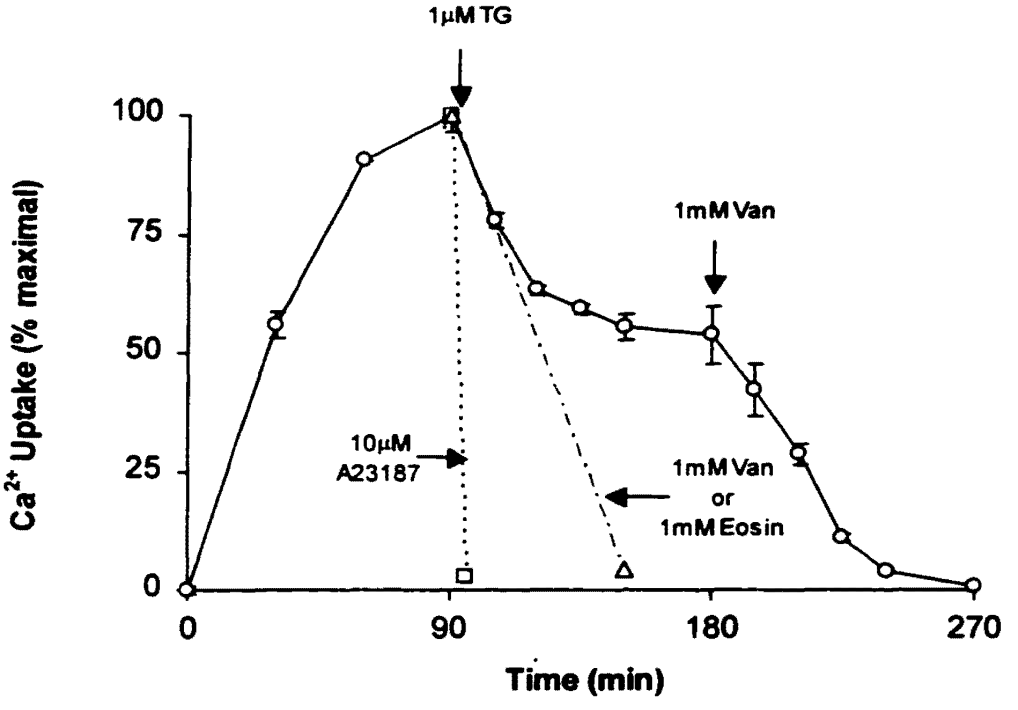
TG-S and TG-I Ca^{2+} sequestering compartments both contain passive efflux pathways

Intracellular Ca^{2+} buffering compartments are capable of accumulating as well as releasing Ca^{2+}. Thus, TG treatment of cultured cells results in a rapid emptying of ER Ca^{2+} stores [19, 83, 107]. This passive Ca^{2+} efflux, or leakage, from intracellular stores is poorly understood but is distinguished from Ca^{2+} efflux mediated by IP_3Rs and RyRs [105]. To determine whether the TG-I Ca^{2+} sequestering compartment exhibits passive Ca^{2+} efflux, we performed kinetic studies in which Ca^{2+} accumulation was allowed to proceed for 90 min at which point 1μM TG was added. Aliquots of the reaction were removed at different times both during the 90 min uptake period and following TG addition. These aliquots were then filtered, washed, and counted for their $^{45}Ca^{2+}$ content. The addition of 1μM TG inhibits SERCA activity and results in a progressive efflux of calcium which was accumulated into the TG-S compartment (Figure 9). A significant portion of the accumulated calcium which does not appear to leak out of brain microsomes can be made to leak out upon the further addition of 1mM vanadate. Similar results were seen with 1mM eosin (data not shown). This presumably represents passive Ca^{2+} efflux from the TG-I compartment. Vanadate and eosin inhibit both TG-I and TG-S Ca^{2+} accumulation and their addition after 90 min of uptake results in efflux of all accumulated Ca^{2+}. Initial rates of efflux appear to be similar from the TG-S and TG-I compartments. A23187 addition rapidly releases (< 5min) all sequestered Ca^{2+} with much faster

kinetics than the spontaneous efflux. These results suggest that the TG-I Ca^{2+} sequestering compartment may be involved in the uptake and release of intracellular Ca^{2+} and may thus play a significant role in buffering cytoplasmic Ca^{2+}.

Figure 9. TG-S and TG-I Ca^{2+} accumulating compartments exhibit similar passive Ca^{2+} efflux pathways. Ca^{2+} flux studies were performed in brain microsomes as described in Methods to compare the passive efflux characteristics of the TG-S and TG-I components. At near saturation of Ca^{2+} accumulation (90 min), maximally effective TG (1μM; 10 nmol/mg) was added to the reaction, which resulted in progressive loss of accumulated Ca^{2+} with the microsomes, presumably due to the passive efflux from TG-S Ca^{2+} compartments. Around 60 min after TG addition, Ca^{2+} accumulation appeared to reach a new steady state, which was approximately 50% of levels at near saturation. Vanadate (1mM) addition to the reaction at 180 min to block all ATPase activity results in release of the remaining Ca^{2+}. When 1mM vanadate or eosin (open triangle) are added at near saturation all Ca^{2+} is passively released over time. Similar initial rates of Ca^{2+} release are seen for all ATPase inhibitor-induced passive efflux phases (TG, vanadate, eosin). Adding 10μM A23187 at near saturation to introduce an artificial leak path for Ca^{2+} results in complete emptying of all sequestered Ca^{2+} within five min.

FIGURE 9


1μM TG
100
75
50
25
0
1mM Van
10μM
A23187
1mM Van
or
1mM Eosin
0
90
180
270
Ca2+ Uptake (% maximal)
Time (min)

IP_3R and RyR channel activators do not promote release from rat brain TG-I compartments

In addition to passive efflux pathways, intracellular ER Ca^{2+} compartments contain specific Ca^{2+} release channels which allow the stimulation of release of the sequestered Ca^{2+} by the second messenger IP_3 (via IP_3R) or by Ca^{2+} itself (via RyR). Although Ca^{2+} is believed to be the endogenous activator of RyR, high millimolar doses of caffeine are used experimentally to activate release via this channel. Two recently described novel second messenger molecules, cADPr and NAADP, have also been proposed to activate release channels located on TG-I compartments of salivary glands cells and sea urchin egg homogenates, respectively [45, 148]. To determine the sensitivity of TG-I and TG-S compartments to activators of IP_3R and RyR, we carried out Ca^{2+} uptake assays in the presence and absence of TG in which were included either 30μM IP_3, 50mM caffeine, 10μM cADPr, or 10μM NAADP. In these assays the added release channel activator holds the channel in an open configuration during the uptake process thus resulting in a ligand-activated leak via a specific channel. The net Ca^{2+} accumulation can thus be assessed for specific receptor-mediated leak pathways. This approach has been used successfully in the past with rat brain microsomes [139]. Ca^{2+} accumulation into the TG-S compartment demonstrates release activated IP_3, caffeine, cADPr, and NAADP (Table 3). The TG-I compartment is insensitive

to all of these activators. Our results suggest that the TG-S and TG-I compartments differ with respect to their expression of Ca^{2+} release channels.

Table 3. Effect of Ca^{2+} Mobilizing Agents on TG-S and TG-I Ca^{2+} Accumulation in Rat Brain Microsomes

	TG-S		TG-I	
Mobilizer	Ca^{2+} accumulation (% of control)	Ca^{2+} released (% of control)	Ca^{2+} accumulation (% of control)	Ca^{2+} released (% of control)
30μM IP_3	62.4 +/- 5.9%	37.6%	105.2 +/- 2.8%	0.0%
50mM Caffeine	39.1 +/- 8.3%	60.9%	117.6 +/- 20.9%	0.0%
10μM cADPr	84.7 +/- 5.9%	15.3%	105.6 +/- 5.9%	0.0%
500nM NAADP	93.8 +/- 4.4%	6.2%	100.4 +/- 3.4%	0.0%

Table 3. TG-S and TG-I compartments are differentiated by sensitivities to Ca^{2+} mobilizing agents. TG-S and TG-I Ca^{2+} accumulating compartments were monitored as previously described, except modified to include known intracellular Ca^{2+} mobilizing agents at listed effective concentrations. Results are means +/- SE of at least three experiments performed. All mobilizers were capable of releasing a subset of the compartments filled by TG-S Ca^{2+} uptake, with caffeine being most extensive. TG-I Ca^{2+} uptake compartments, however, were not stimulated to release Ca^{2+} by any of these agents.

The TG-I Ca^{2+} accumulation is widely distributed in different brain regions, different brain cell types, and neuronal and glial cell lines

TG-I Ca^{2+} sequestration in rat brain microsomal preparations was previously shown to be differentially expressed in distinct brain regions with higher levels seen in the brain stem and spinal cord relative to striatum and cerebral cortex [139]. We find similar differences in regional microsomes prepared from pig brain stem and spinal cord (Table 4). Pig spinal cord has greater TG-I Ca^{2+} accumulation than brain stem, followed by cerebellum and forebrain. These findings suggest that TG-I Ca^{2+} accumulation is an evolutionarily conserved regionally selective property of mammalian brain. Since brain microsomes represent subcellular compartments from many different cell types, we performed experiments in rat brain primary cell cultures of cerebellar granule cells and whole brain astrocytes. Both of these cell types demonstrate TG-I Ca^{2+} accumulation with the neuronal granule cells having three times the amount demonstrated by the glial astrocyte cells (Table 4). While these cell types represent only a sampling of brain cell types, the higher levels of TG-I Ca^{2+} accumulation in neuronal cells (see also below) may indicate a unique role for this process in neuronal function. Several different cancer cell lines have been reported to possess TG-I Ca^{2+} accumulating compartments including the rat pheochromocytoma PC-12 cell line derived from adrenal medulla [113]. To see if human brain derived cells also express TG-I Ca^{2+} accumulation, we compared the level of TG-I Ca^{2+} accumulation seen in PC-12

cells to that in two different human neuronal cell lines and two different human glial cell lines. For these studies we employed cell pellet homogenates in the presence of 10μM digitonin. This concentration of digitonin effectively permeabilized the plasma membrane of >95% of cells as determined by trypan blue labeling of PC-12 cells and U-87 human glioblastoma cells (Figure 10A). Even 10-fold higher concentrations of digitonin had no effect on either TG-S and TG-I Ca^{2+} accumulation in rat brain microsomes (Figure 10B) or cell homogenates (data not shown). Thus, plasma membrane vesicles do not contribute to the accumulated calcium signal in these studies. In the SH-SY5Y human neuroblastoma cell line, 23.1% of the total Ca^{2+} accumulation is mediated by the TG-I component as compared to 29.8% in PC-12 cells. In the NBFL human neuroblastoma cell line (developed and kindly provided by Dr. Aviva Symes), only 14.1% of the total Ca^{2+} accumulation is accounted for by the TG-I component. In the U-251 glioblastoma cell line only 4.4% of total Ca^{2+} accumulation is sequestered by the TG-I component while this component accounts for half of the total Ca^{2+} accumulation (49.1%) in U-87 glioblastoma cells. These findings demonstrate that cells derived from human brain also contain TG-I Ca^{2+} accumulation with widely varying proportions of TG-S and TG-I being expressed in different cell lines.

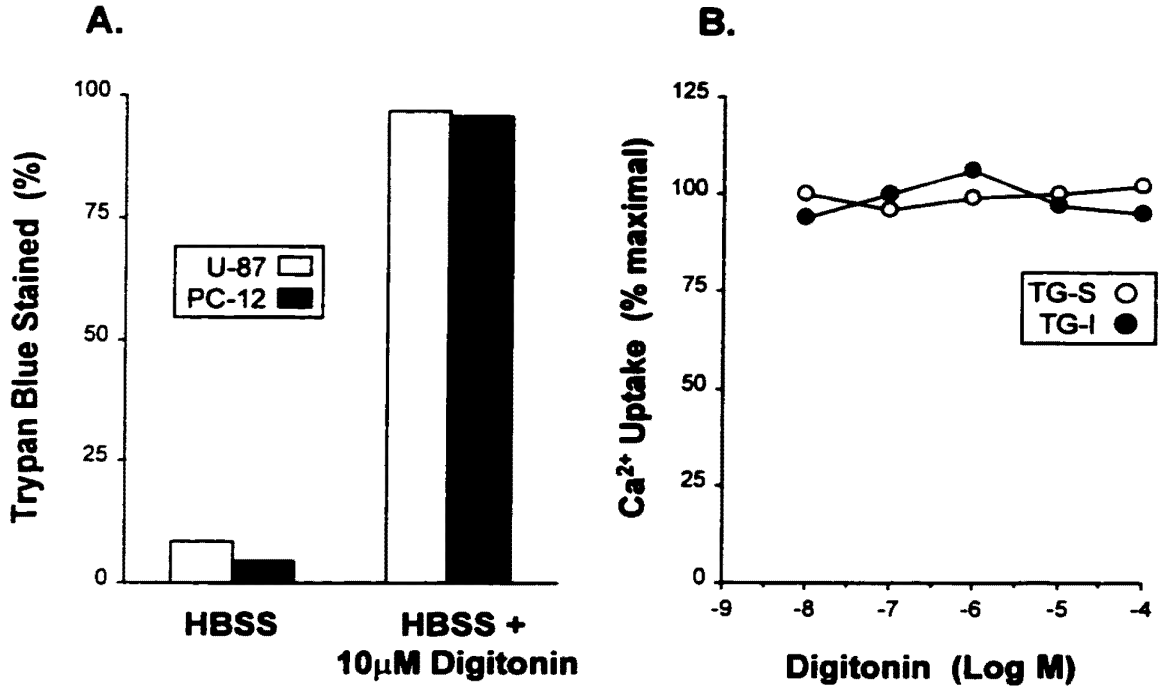
Table 4. TG-I Ca^{2+} is widely and variably distributed in brain-derived microsomes or crude homogenates from different species, cell types, and cell lines. TG-S and TG-I Ca^{2+} uptake were analyzed as described in the indicated brain-derived tissue and cell preparations, and percent TG-I of total Ca^{2+} accumulation reported. Mean TG-I Ca^{2+} uptake +/- SE from at least three independent experiments is shown. TG-I Ca^{2+} uptake in pig brain microsomes displays regional variation with higher levels in hindbrain structures. Also, TG-I Ca^{2+} uptake among two different cell types of the rat brain differ markedly, as shown by the three-fold increase in the TG-I activity of primary cerebellar granule cell cultures as compared to whole brain astrocytes. Cancer cell lines display a wide variation in the TG-I component of the overall Ca^{2+} uptake, from a high measured at almost 50% total Ca^{2+} uptake activity in highly malignant U-87 human glioblastoma cells to a very low percent seen in a different glioblastoma cell line, U-251 cells. Total Ca^{2+} uptake, however, was similar amongst the cell lines tested.

Table 4. TG-I Ca^{2+} Accumulation in Different Tissue Preparations

Tissue Preparation	TG-I Ca^{2+} Accumulation (% of total)
Rat Whole Brain Preparations:	
Crude homogenates	29.6 +/- 6.8%
Microsomes	20.8 +/- 4.8%
Pig Regional CNS microsomes:	
Spinal Cord microsomes	24.2 +/- 7.1%
Brainstem microsomes	17.6 +/- 1.8%
Cerebellum microsomes	12.1 +/- 2.0%
Forebrain microsomes	11.7 +/- 1.2%
Rat Brain Primary Cell Cultures:	
Cerebellar Granular Cells	19.7 +/- 5.6%
Whole Brain Astrocytes	6.4 +/- 2.9%
Cultured Cell Lines:	
U-87 (Glioblastoma, human)	49.1 +/- 6.1%
PC-12 (Pheochromocytoma, rat)	29.8 +/- 5.4%
SH-SY5Y (Neuroblastoma, human)	23.1 +/- 0.1%
NBFL (Neuroblastoma, human)	14.1 +/- 1.6%
U-251 (Glioblastoma, human)	4.4 +/- 0.8%

Figure 10. TG-S and TG-I Ca^{2+} accumulation is not sensitive to digitonin permeabilization of plasma membrane. Low concentrations of digitonin preferentially and effectively permeabilize cells as measured by trypan blue staining (**A**). Six well plates containing U-87 and PC-12 cells were incubated for 30 min in HBSS in the absence and presence of 10μM digitonin. Trypan blue exclusionary dye was used to measure plasma membrane permeabilization. Values represent means +/- SE of cells from duplicate wells counted in triplicate. While staining in the presence of HBSS alone was under 10%, HBSS with 10μM digitonin effectively permeabilizes ~ 95% of U-87 and PC-12 cells. B. Digitonin has no effect on microsomal, or brain homogenate (data not shown), Ca^{2+} accumulation at concentrations up to ten-fold greater (100μM) than that which effectively permeabilized plasma membrane of cell cultures. Maximal $^{45}Ca^{2+}$ uptake was considered uptake in the absence of digitonin. Results indicate that plasma membrane revesicularization along with its resident PMCAs does not contribute significantly to TG-S and TG-I Ca^{2+} accumulation in our microsome preparation.

FIGURE 10


A.
Trypan Blue Stained (%)
100
75
50
25
0
U-87
PC-12
HBSS
HBSS +
10μM Digitonin
B.
Ca2+ Uptake (% maximal)
125
100
75
50
25
0
TG-S
TG-I
-9
-8
-7
-6
-5
-4
Digitonin (Log M)

The TG-I Ca^{2+} sequestering compartment is enriched in rat nervous tissue and is selectively expressed in distinct brain regions

To determine the expression of the TG-I Ca^{2+} accumulating compartment in normal rat tissues, we employed $^{45}Ca^{2+}$ uptake assays in fresh frozen sections of several different rat tissues. Fresh frozen sections have previously been shown to accumulate $^{45}Ca^{2+}$ in an ATP- and Mg^{2+}-dependent manner with the uptake selectively representing sequestration by ER [139, 140]. The sections are simply substituted for microsomes or homogenates in uptake assays and the results can be analyzed as either radioactive counts accumulated by the sections or by autoradiography to localize the accumulated signal to distinct anatomical compartments. As before, these assays contain 10μM digitonin to permeabilize plasma membranes. For anatomical localization, best results were empirically obtained using buffers containing a combination of oxalate and chloride as the co-transported anions. The TG-I Ca^{2+} accumulation can reliably be measured in tissue sections of rat brain and other organs using the oxalate-chloride combined anion buffer as shown in Table 5. The TG-I component was determined by including TG (10 nmol/mg protein) in the uptake assays. About 20% of the total Ca^{2+} accumulation in rat brain sections is accounted for by the TG-I component. Similar results were also seen when 500μM BHQ or CPA were utilized (data not shown). Among the many peripheral organs screened, the adrenal gland had the next highest level of TG-I Ca^{2+} accumulation (18.5%) as a percent of total Ca^{2+} accumulation. In cardiac

muscle, 12.6% of the total uptake was TG-insensitive while in skeletal muscle, hardly any TG-I component (1.7%) could be detected. Many other organs display a TG-I component at intermediate levels ranging from 8.9% in the kidney to 0% in bladder (Table 5). These results suggest that the unique anatomical expression of TG-I Ca^{2+} accumulation may be associated with distinct organ functions and further supports an important role for this process in nervous tissue.

Table 5. TG-I Ca^{2+} uptake accounts for a much greater proportion of Ca^{2+} uptake activity in rat brain than in other organs. Energy dependent Ca^{2+} uptake was performed using fresh frozen sections of several rat tissues in place of microsomes as described in Methods. Total accumulated $^{45}Ca^{2+}$ in sections was determined by scraping sections from the slides and measuring radioactivity. Serial sections were subjected to treatment with A23187 (100nmol/mg) and TG (10nmol/mg) for the determination of the non-specific and TG-I signal, respectively. Serial sections were also processed through the uptake and wash procedures but were used for the determination of protein using concentrated protein dye as described. At least 3 serial sections were used for each treatment and for protein determination and less than a 3% difference was observed in values obtained from serial sections within a treatment group. Highest levels and proportions of TG-I were found in brain followed by the adrenal gland. The heart displayed intermediate levels while other organs had much lower activity, with the bladder being the lowest.

Table 5. TG-I $^{45}Ca^{2+}$ Accumulation in Rat Brain and Peripheral Organs

Organ	TG-I Ca^{2+} Accumulation (% total accumulation)
Brain	20.3 +/- 1.6%
Adrenal gland	18.5 +/- 1.8%
Heart	12.6 +/- 2.5%
Kidney	8.9 +/- 1.0%
Lung	8.1 +/- 0.8%
Thymus	6.8 +/- 1.7%
Liver	5.8 +/- 1.6%
Spleen	5.2 +/- 0.4%
Uterus	5.1 +/- 3.3%
Large Intestine	4.8 +/- 1.4%
Eye	4.6 +/- 2.0%
Pancreas	2.5 +/- 0.5%
Skin	2.1 +/- 0.3%
Skeletal muscle	1.7 +/- 1.1%
Bladder	0.0 +/- 0.3%

Analysis of $^{45}Ca^{2+}$ accumulation in tissue sections via autoradiography reveals unique anatomic compartmentation of this process within different organs. In the adrenal gland the majority of accumulated Ca^{2+} is seen in the medulla, which is enriched in sympathetic neurons (data not shown). Ca^{2+} uptake is also seen at lower levels in the adrenal cortex with the zona granulosa having greater accumulation than other layers (data not shown). The TG-I component of total Ca^{2+} accumulation by adrenal gland sections is predominantly associated with the medulla (data not shown). In rat brain sections, total Ca^{2+} accumulation shows remarkable regional heterogeneity with distinct enrichment in specific cell layers, such as cerebral cortex, hippocampus, striatum, and cerebellar cortex (Figure 11A and B). TG selectively inhibits Ca^{2+} accumulation into a number of these regions, including: cerebral cortex, striatum, and cerebellum. Certain brain regions, however, are relatively insensitive to TG inhibition at doses that maximally inhibit TG-S (see above). Ca^{2+} accumulation in these regions thus represents the TG-I Ca^{2+} pool. This compartment is highly enriched in the thalamus, the superior and inferior colliculi, brain stem, pontine nuclei, and deep cerebellar nuclei. Areas demonstrating very little TG-I Ca^{2+} accumulation include: striatum, hypothalamus, substantia nigra, olfactory tubercle, and basal forebrain. While the cerebral cortex demonstrates both TG-S and TG-I Ca^{2+} accumulation, the patterning of these two processes appears to be distinct. Thus, the TG-S component is selectively enriched in the superficial cortical layers, while TG-I is enriched in the peri-collosal, deep cerebral cortical layers. In the hippocampus,

TG-S and TG-I components are found in the pyramidal and dentate gyrus cell layers. In the cerebellum, very high levels of TG-S are associated with the cortical layers with highest levels in the Purkinje cell layer followed by the molecular layer. The granule cell layer and deep cerebellar nuclei display lower levels of total Ca^{2+} accumulation. The highest TG-I components of cerebellar Ca^{2+} accumulation are associated with the deep cerebellar nuclei followed by the Purkinje cell layer. Molecular and granule cell layers have lower TG-I Ca^{2+} accumulation. These results reveal, for the first time, a novel compartmentation of TG-S and TG-I Ca^{2+} pools in rat brain. The highly unique expression patterns for the TG-I pool observed in our studies imply a very important regionally selective function for this process in the mammalian brain.

Figure 11. Rat brain Ca^{2+} sequestering compartments with distinct sensitivities to thapsigargin are anatomically segregated. Mg^{2+}/ATP-dependent $^{45}Ca^{2+}$ accumulation was performed in the absence and presence of 1uM TG or 10uM A23187 using fresh-frozen rat brain sections in place of microsomes and a buffer containing oxalate and chloride as described in Materials and Methods. Sections were analyzed via autoradiography to localize the $^{45}Ca^{2+}$ accumulating compartments anatomically. The coronal brain sections displayed proceed in a rostro-caudal direction. $^{45}Ca^{2+}$ accumulation is demonstrated by many structures of the brain, is generally much higher in grey than white matter, and is abolished by A23187. TG produces a marked inhibition of $^{45}Ca^{2+}$ accumulation in many brain structures. However, many brain regions demonstrate TG-resistant $^{45}Ca^{2+}$ accumulation. While in some structures Ca^{2+} accumulation is composed of both TG-S and TG-I components, other regions appear to exclusively express either TG-S or TG-I. Thus, the cerebral cortex of the frontal pole (FRP) in panel A displays much more TG-S than TG-I while unique sensitivities of the main olfactory bulb (MOB) and anterior olfactory nucleus (AON) to TG are apparent. The MOB granule cell layer (MOBgr) and outer plexiform layer (MOBopl) appear to be enriched in TG-I. Within the cerebral cortex a unique layering of the TG-I component is apparent, as seen in panels B and C. The cerebral cortical layers are indicated in panel B and the TG-I components appears most enriched in layer 6 followed by layer 4. In the piriform area (PIR), however, layer 1 ($PIR_{(1)}$) is most enriched in TG-I. The taenia tecta (TT), caudate-putamen (CP), olfactory tubercle (OT), and nucleus accumbens (ACB) have much higher TG-S than TG-I while the claustrum (CLA) and septum (S) have somewhat higher porportions of TG-I than these structures. In panel D, the hippocampus (HIP) and thalamus (TH) both display prominent $^{45}Ca^{2+}$ accumulation with significant heterogeneity seen amongst subregions. In the CA1-CA3 layers of the HIP, the pyramidal cell bodies account for the majority of the uptake while in the dentate gyrus (DG) granule cell bodies make up the major signal. All hippocampal layers appear to have both TG-I and TG-S components. Many thalamic nuclei accumulate $^{45}Ca^{2+}$ and the TG-I appears to be more enriched in the lateral geniculate (LG) body, the venteropostero-lateral (VPL) and venteropostero-medial (VPM) nuclei with lower proportions in the reticular thalamic (RT) nucleus, zona inserta (ZI), and the subthalamic nucleus (STN). The amygdala nuclei are predominantly TG-S. In panel E, the substantia nigra (SN) and ventral tegmental area (VTA) display more TG-S than TG-I while distinct enrichment of TG-I are seen in the medial geniculate (MG), red nucleus (RdN), and specific areas of the superior colliculus (SC) such as the deep grey region (SCdg). In panels F-G, the TG-S and TG-I appear to become more distinctly segregated. Thus, while cerebellar cortex (CBC) is rich in TG-S, panel F reveals that TG-I predominantly counts for the $^{45}Ca^{2+}$ accumulation in the inferior colliculus (IC), nucleus of the lateral lemniscus (NLL), rostral pontine reticular nucleus (PRNr), and pontine grey (PG). Similarly, in panels G and H TG-I appears to primarily count for the $^{45}Ca^{2+}$ accumulation into the dorsal tegmental nucleus (DTN), ventral cochlear nucleus (VCO),

trigeminal nucleus (V), principle sensory nucleus of the trigeminal (PSV), the olivary complex (OLV), the nucleus raphe magnus (RM), deep cerebellar nuclei (DCBN), spinal vestibular nucleus (SPIV), dorsal cochlear nucleus (DCO), medial vestibular nucleus (MV), and the collection of nuclei making up the reticular formation (RN). The choroid plexus (chp) contains both TG-S and TG-I components. White matter bundles including the inferior cerebellar peduncle (icp), spinal tract of the trigeminal nucleus (sptV), pyramidal tract (py), cortical spinal tract (cs), cerebral peduncles (cp), anterior commissure (aco), and corpus collosum (cc) display much lower levels of $^{45}Ca^{2+}$ accumulation but appear to have both TG-S and TG-I components.

FIGURE 11, A-D

Total $^{45}Ca^{2+}$ uptake	+ Thapsigargin (1uM)	+ A23187 (10uM)

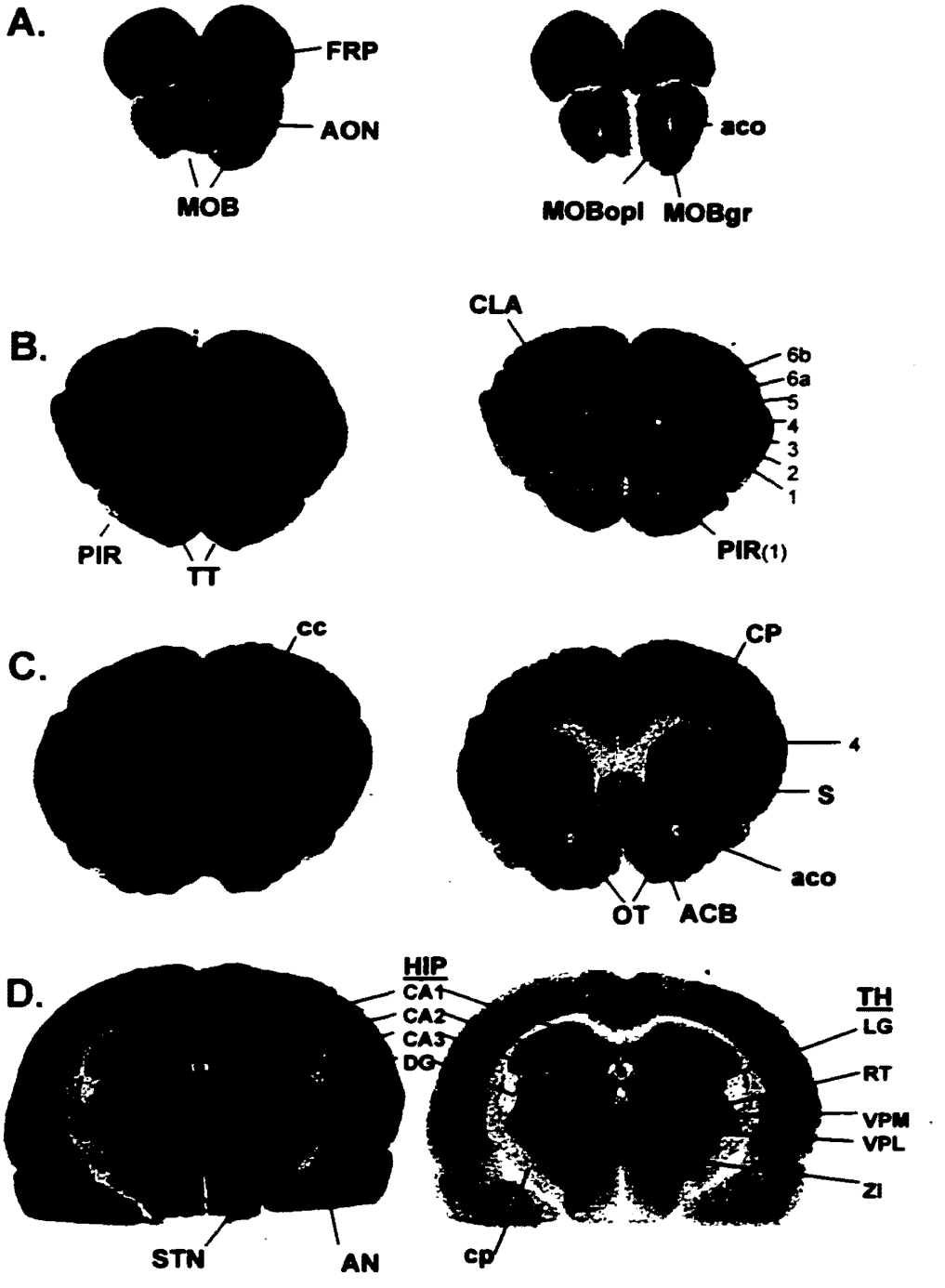
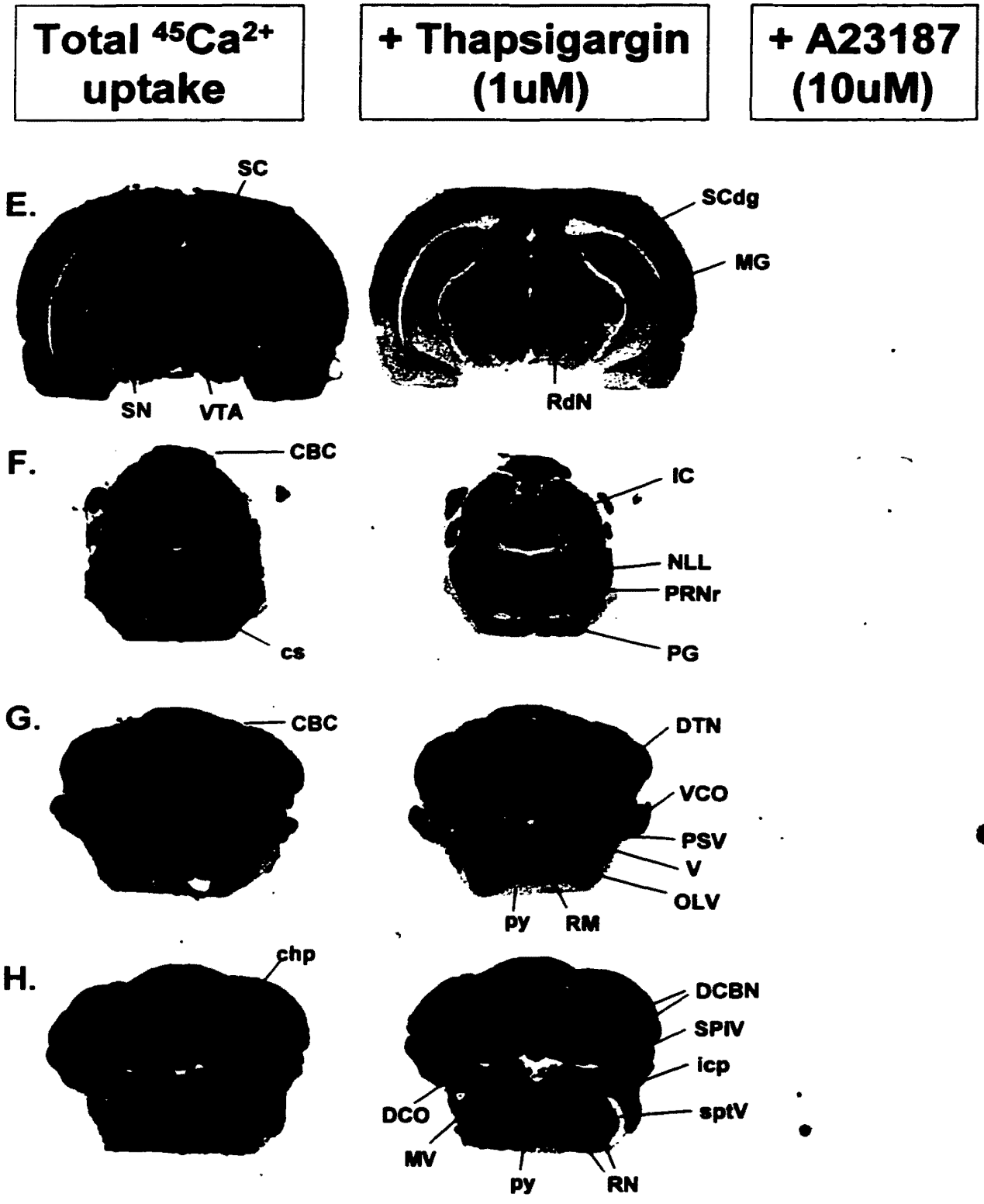


FIGURE 11, E-H


Total $^{45}Ca^{2+}$ uptake
+ Thapsigargin (1uM)
+ A23187 (10uM)
E.
SC
SCdg
MG
SN
VTA
RdN
F.
CBC
IC
NLL
PRNr
cs
PG
G.
CBC
DTN
VCO
PSV
V
OLV
py
RM
H.
chp
DCBN
SPIV
icp
DCO
sptV
MV
py
RN

DISCUSSION

We have characterized a novel Ca^{2+} sequestering compartment which is > 1000-fold less sensitive to inhibition by TG, a compound routinely employed as a potent inhibitor of all known SERCA isoforms. TG-I Ca^{2+} accumulation by rat brain preparations is primarily associated with microsomal preparations that are generally employed for studies involving ER functions. Rat brain microsomal Ca^{2+} accumulation requires the presence of anions which are co-transported with the actively sequestered Ca^{2+}. Very little is known about the nature of ER-associated anion transporters, although recent studies have identified intracellular chloride [112] and phosphate anion channels [74, 93, 102] which may be involved in this process. Most studies involving SR or ER Ca^{2+} accumulation employ either chloride, phosphate, or oxalate as the co-transported anion. The results shown in Figure 2 demonstrate that the choice of anion employed in uptake buffers can markedly change the proportion of Ca^{2+} sequestration mediated by TG-S and TG-I components. While oxalate and phosphate-supported uptake reveals both TG-S and TG-I components, the proportion of the TG-I compartment is greater with phosphate. With phosphate buffers, the relative contribution of TG-S and TG-I to the total Ca^{2+} accumulation also varies as a function of assay times (Figure 2C). Thus, the TG-I pool appears to fill more rapidly and represents a higher proportion of overall uptake when examined prior to saturation. Since studies of Ca^{2+} transport vary in terms

of both assay buffer anions and incubation times, the relative contribution of TG-I and TG-S to overall uptake may not have been fully appreciated previously. Chloride-supported uptake primarily favors accumulation into the TG-I compartments (Figure 2D). Phosphate and oxalate form complexes with the concentrated Ca^{2+} within the lumen of Ca^{2+} accumulating vesicles, and their ability to enhance Ca^{2+} uptake based on this property has generally been suggested as the reason for their use in Ca^{2+} transport studies. Our findings suggest that unique anion permeabilities are also associated with TG-S and TG-I compartments. The TG-S compartment apparently displays relative differences in anion permeability with oxalate > phosphate > chloride. Our findings thus reveal important methodological principles fundamental to Ca^{2+} transport studies. Oxalate is not a physiological anion and the predominant endogenous anion participating in Ca^{2+} co-transport is likely to be phosphate. While the TG-I component of phosphate-supported Ca^{2+} uptake in whole brain microsomes represents about 25% of the total at saturation, its physiological contribution may be much greater in non-saturating conditions and in distinct brain regions.

The mechanism of Ca^{2+} transport by the TG-I component of brain microsomes is unknown. We have shown that the TG-I Ca^{2+} accumulation has an absolute requirement for ATP and Mg^{2+} (Figure 4), suggesting the involvement of an ion-motive ATPase. The optimal concentrations of ATP,

Mg^{2+}, and also Ca^{2+} for both TG-S and TG-I uptake appear to be similar (Figure 5A-C). This suggests that both TG-S and TG-I uptake mechanisms would be mutually active under similar intracellular conditions. The TG-I component is also insensitive to inhibition by the SERCA inhibitors BHQ and CPA (Figure 7), strongly suggesting that the mechanism involved in Ca^{2+} accumulation into this compartment does not involve a known mammalian SERCA isoform. Both the TG-S and TG-I mechanisms are inhibited by the broad ATPase inhibitors vanadate and eosin (Table 2). Vanadate inhibition is believed to be a relatively unique feature of P-type ATPases [109]. Several different mammalian ion-motive P-type ATPases in addition to SERCAs are known to transport specific cations. These include the Na^+/K^+-ATPase, the H^+/K^+-ATPase, the PMCA, as well as the Cu^{2+}-transporting ATPases known as the Menke's and Wilson's disease gene products. It is possible that the TG-I components observed in our assays is in some way associated with one of these activities. Thus, transport of Na^+, K^+, or H^+ ions may be coupled with a cation exchange mechanism involving Ca^{2+} to generate the TG-I Ca^{2+} accumulation. We have employed pharmacological strategies to address these possibilities. Ouabain and omeprazole, specific inhibitors of the Na^+/K^+-ATPase and the H^+/K^+-ATPase, respectively, had no effect on TG-I Ca^{2+} accumulation. Furthermore, FCCP, a hydrogen ionophore, and monensin, a H^+/Na^+ ionophore, were also without effect. These treatments, at the doses utilized, would be expected to eliminate the ion-motive force driving secondary Ca^{2+} transport. The PMCA can be potently inhibited by vanadate and eosin, however, these inhibitors are not

specific for this P-type ATPase. It has been shown that the PMCA is inhibited by vanadate at concentrations below 10μM, while the SERCAs are not inhibited under this condition. This criterion has, in fact, been used to distinguish these enzyme activities [22]. In our studies, we determined the apparent IC_{50}'s for vanadate inhibition of the TG-S and TG-I Ca^{2+} accumulation to be ~ 100μM (Table 2). Eosin was also found to have similar potencies in inhibiting the TG-S and TG-I Ca^{2+} accumulation with apparent IC_{50}s of about 10μM (Table 2). Inhibition of TG-I Ca^{2+} accumulation by these inhibitors thus can not specifically implicate involvement of the PMCA in this process. Calmodulin is known to stimulate several isoforms of the PMCA at sub-micromolar levels, but had no effect in our Ca^{2+} transport assays at concentrations of 10μM (data not shown) [21]. Furthermore, PMCA is a component of the plasma membrane and all of our assays routinely included 10μM digitonin which selectively permeabilizes the plasma membrane. A large majority of the studies involving TG-I $^{45}Ca^{2+}$ accumulation in cultured cell lines also employ digitonin-permeabilized cells. No specific inhibitors are known for the recently identified Cu^{2+}-transporting mammalian P-type ATPases. Yeast possess a Mn^{2+}-transporting P-type ATPase and a mammalian homologue has also recently been identified [30]. It is possible that one of these mechanisms may be responsible for the TG-I Ca^{2+} accumulation with Ca^{2+} serving as a surrogate cation for these transport mechanism. If this is the case, however, then these processes may still regulate intracellular Ca^{2+} homeostasis since in all of our assays the free Ca^{2+}

concentration was carefully titrated to reflect normal baseline cytoplasmic free Ca^{2+} levels. We can not currently exclude the possible involvement of these newly discovered P-type ATPases in TG-I Ca^{2+} accumulation.

Although not conclusive, TG-I Ca^{2+} accumulation appears to represent an intracellular compartment. The relatively minimal loss of TG-I Ca^{2+} accumulation in brain microsomes as compared to crude homogenates lends support to the theory that TG-I activity is an ER-like intracellular compartment. Additionally, as stated above, digitonin was unable to effect TG-I Ca^{2+} uptake in microsomal (or homogenate) preparations suggesting that the membrane composition of the TG-I Ca^{2+} accumulation compartment is distinct from that of the plasma membrane. The use of sodium azide (a mitochondrial ATPase inhibitor), low nanomolar free Ca^{2+} in the assay buffer (well below the affinity of mitochondria Ca^{2+} transporter), and freeze-thawed tissue preparations (which disrupts mitochondrial function), all tend to eliminate mitochondrial contribution to TG-I Ca^{2+} accumulation under our assay conditions. However, the exclusion of mitochondrial involvement does not automatically provide evidence that the ER is the source of TG-I Ca^{2+} accumulation. Furthermore, the extensive pharmacology (Table 2) used to examine the TG-I component cannot assign or exclude the identify a specific subcellular TG-I compartment. To specifically identify this compartment will require more intense subcellular techniques, such as immunohistochemistry and confocal microscopy. The isolation of the TG-I

compartment would be a major advancement in this field as marker studies, immunoprecipitation, etc., could then be used to identify the compartment and its parent organelle.

The requirement of the TG-I Ca^{2+} accumulation for Mg^{2+}/ATP as well as its inhibition by vanadate strongly suggests ATPase activity with the involvement of a P-type ATPase. To explore an association of TG-I Ca^{2+} accumulation with ATPase activity we simultaneously monitored Ca^{2+}-stimulated, Mg^{2+}/ATP-dependent TG-S and TG-I $^{45}Ca^{2+}$ accumulation and inorganic phosphate release. In rat brain microsomes, Ca^{2+} simultaneously stimulated both $^{45}Ca^{2+}$ accumulation and phosphate release from ATP in a dose dependent manner (Figure 8A). Two distinct $^{45}Ca^{2+}$ uptake and ATPase activities with similar Ca^{2+}-dependent profiles could be distinguished by their sensitivities to TG. This suggests that the TG-I Ca^{2+} accumulation is indeed associated with Ca^{2+}-stimulated ATPase activity. We also explored whether a Ca^{2+}-stimulated phospho-enzyme intermediate, characteristic of P-type ATPases, was associated with TG-I Ca^{2+} accumulation. Phospho-enzyme intermediate formation can only be detected by slowing down the ATPase activity at 4 °C. Since no Ca^{2+} transport occurs under these conditions, we determined whether we could detect Ca^{2+}-stimulated phospho-enzyme activities with differential sensitivities to the SERCA inhibitors TG, BHQ, and CPA. Rat brain microsomes incubated with [γ^{32}P] ATP demonstrated a Ca^{2+}-stimulated

incorporation of [^{32}P] into TCA precipitable proteins at 4 °C (Figure 8B and C). No signal was seen in the presence of 5mM EGTA. Concentrations of TG (500nM), BHQ (750μM), and CPA (500μM) which completely inhibit SERCA activity showed incomplete inhibition of the Ca^{2+}-stimulated incorporation of [^{32}P] into TCA precipitable proteins. Approximately 25% of the phospho-enzyme signal, as determined by scintillation counting of aliquots, was insensitive to the SERCA inhibitors. When these TCA precipitated, radiolabeled fractions were analyzed by acidic gel electrophoresis, a major radioactive band was seen at approximately 110 kDa by autoradiography. Although some low molecular weight diffuse (< 20 kDa) radioactive signals were also seen, these did not form a consistently recognized, distinct protein band. ^{32}P-labeling of the 110 kDa band was abolished by 5mM EGTA but was incompletely inhibited by the SERCA inhibitors. Our findings demonstrate a TG-insensitive Ca^{2+}-stimulated Mg^{2+}/ATPase activity and a Ca^{2+}-stimulated phospho-enzyme intermediate which is associated with the TG-I Ca^{2+} accumulation. It is tempting to speculate that the 110 kDa ^{32}P-labeled band seen in the presence of SERCA inhibitors is also responsible for the Ca^{2+}-stimulated ATPase activity and the TG-I Ca^{2+} accumulation. However, the identity of a protein mediating these associated activities must await further studies. While the TG-I Ca^{2+} accumulation, the TG-I Ca^{2+}-stimulated Mg^{2+}/ATPase activity and the TG-I Ca^{2+}-stimulated phospho-enzyme formation are all not inhibited by known SERCA inhibitors this does not rule out the

possibility that a novel SERCA isoform may be responsible for these activities. The sensitivity of SERCAs to TG depends on a critical stretch of 6 amino acid residues in the S3 stalk segment [151]. In fact, the substitution of a single amino acid within this region (Gly 257 to Ile) results in significant loss of sensitivity to TG. CPA was found recently to be sensitive to the same S3 stalk segment mutation [85]. Similar studies have been not performed for BHQ. While it is conceivable that such a mutation may arise spontaneously in cancer cell lines, this is unlikely to be the case in normal rat brain. We therefore speculate that a novel SERCA isoform, yet to be discovered, which differs from other SERCA isoforms in the S3 stalk segment, may account for the TG-I Ca^{2+} uptake. Indeed, a novel SERCA isoform was recently discovered in paramecium that displays a unique intracellular localization and approximately 50% homology with mammalian isoforms and is insensitive to TG inhibition [54, 72].

Intracellular Ca^{2+} buffering compartments participate in both uptake and release activities. ER preparations are known to contain both non-specific, or passive, Ca^{2+} leak pathways as well as specific Ca^{2+} release pathways involving known second messenger activated channels. While the passive leak pathways are poorly understood, the specific release pathways are mediated by various isoforms of the IP_3R and RyR. To evaluate whether a passive Ca^{2+} leak mechanism is associated with the TG-I Ca^{2+} pool, we performed kinetic

Ca^{2+} flux studies in which phosphate-supported Ca^{2+} uptake into brain microsomes was allowed to reach near saturation. At this point, TG at a dose which maximally inhibits SERCA activity was added to the assay. Under these conditions we observed a progressive loss of accumulated Ca^{2+} from the microsomal vesicles which presumably represents passive Ca^{2+} efflux from the TG-S Ca^{2+} pool (Figure 9). This observation may signify that spontaneous release activity via such an efflux pathway occurs continuously during the uptake assay but is masked by the re-sequestration of Ca^{2+} via ion-motive ATPases. The progressive loss of accumulated Ca^{2+} upon SERCA inhibition also challenges the prevailing concept that Ca^{2+} and phosphate form intraluminal insoluble precipitates in ER vesicles [52, 61]. The initial rapid rate of Ca^{2+} efflux following TG inhibition appears to slow progressively to reach a new steady state by ~ 90 min post TG addition, which represents 50% of the accumulated Ca^{2+} at near saturation. To determine whether the remaining sequestered Ca^{2+} represented uptake by the TG-I Ca^{2+} pool, we then added 1mM vanadate to the assay to block the TG-I Ca^{2+} sequestering mechanism. This resulted in another phase of the accumulated Ca^{2+} being released from the microsomes. This second Ca^{2+} efflux appears to represent passive release from the TG-I pool, as it resulted in complete emptying of accumulated microsomal Ca^{2+}. When 1mM vanadate (or 1mM eosin) was added at near saturation, a single phase of release resulting in complete loss of sequestered Ca^{2+} was observed. The initial rates of release observed when either TG, vanadate, or eosin was added at near saturation or when vanadate was added

subsequent to depletion the TG-S pool were very similar. This may suggest that a common release or passive efflux mechanism for Ca^{2+} exists on both the TG-S and TG-I compartments. When an artificial Ca^{2+} leak pathway was introduced into the microsomal membranes via the addition of A23187 at near saturation, a much more rapid loss of accumulated Ca^{2+} was observed with complete emptying of microsomes within 5 min. Our findings have important implications for all studies that utilize TG to deplete intracellular Ca^{2+} pools. While TG treatment of cells and tissues would be expected to induce passive efflux from the TG-S compartment, a significant proportion of this release Ca^{2+} may be subsequently sequestered within the TG-I pool. Changes in cytoplasmic Ca^{2+} levels under these conditions would thus reflect the combined activities of TG-S efflux and TG-I accumulation. Emptying of TG-S Ca^{2+} pools by TG treatment (presumably mediated by passive efflux) is known to elicit Ca^{2+} influx from the extracellular environment in an attempt to refill the depleted Ca^{2+} stores. This phenomenom, known as store operated or capacitative Ca^{2+} entry, remains poorly understood. A major unresolved issue involves the identity of the compartment where capacitative Ca^{2+} entry is sequestered. Since this entry takes place in the presence of TG, we propose that it is in fact the TG-I pool which buffers the rise in cytoplasmic Ca^{2+} produced by capacitative influx.

To determine whether specific second messenger activated Ca^{2+} release channels were associated with the TG-I Ca^{2+} compartment, we determined the

relative sensitivities of net TG-S and TG-I Ca^{2+} uptake to IP_3, caffeine, cADPr, and NAADP, which are known activators of intracellular Ca^{2+} release channels. As shown in Table 3, all of these agents were able to act on the TG-S Ca^{2+} pool, but were without effect on the TG-I Ca^{2+} pool. These results demonstrate that while the TG-S and TG-I Ca^{2+} pools are both capable of energy dependent Ca^{2+} accumulation as well as passive Ca^{2+} efflux, they are differentially regulated by intracellular Ca^{2+} mobilizing agents. Previously studies which have explored Ca^{2+} release from the TG-I pool in cultured cell lines have presented inconsistent findings. For example, the TG-I pool was found to be sensitive to IP_3 in rat and dog salivary gland cells, and DC-3F/TG-2 cells, and it was sensitive to caffeine in rat ventricle isolates and GH-3 cells (see Table 1). Cyclic ADP ribose was reported to release Ca^{2+} from the TG-I pool in dog salivary gland cells, while NAADP acted on the TG-I pool in sea urchin egg homogenates (see Table 1). Several other studies, however, found no effect of Ca^{2+} release agents on the TG-I pool (see Table 1). Since the majority of these studies were performed in cancer cell lines, it is difficult to draw conclusions which would apply to normal organs. However, the action of cADPr on microsomes of the dog salivary gland may suggest that the TG-I pool is differentially regulated in different organs. It is also possible that specific Ca^{2+} release agents acting on the TG-I pool are yet to be fully appreciated [50]. The lack of effect of Ca^{2+} releasers on the TG-I pool also does not diminish its potential importance for regulating cell biology. Mitochondria, for example,

participate in Ca^{2+} buffering via uptake and release but are not sensitive to a known physiologic Ca^{2+} mobilizing agent.

Studies describing a TG-I Ca^{2+} pool have been performed in a variety of different cell lines and a few isolated mammalian organ preparations (see Table 1). Many of these studies have inferred the existence of a TG-I Ca^{2+} pool by studying changes in cytoplasmic Ca^{2+} concentrations with fluorescent dyes rather than by direct determination of TG-I Ca^{2+} sequestration. Studies examining TG-I Ca^{2+} sequestration using $^{45}Ca^{2+}$ have employed widely varying buffers. It is thus difficult to determine whether the widely varying proportions and properties of the TG-I pool reported in the literature reflect differences in assay techniques or true differences in the TG-I pool among different tissue and cell types. Having determined optimal assay conditions for distinguishing TG-S and TG-I Ca^{2+} pools, we have explored TG-I Ca^{2+} accumulation in regional brain preparations, primary brain cell cultures, and brain cell-derived cancer cell lines. In the very first description of a TG-I Ca^{2+} pool, Verma *et al.* described marked differences in TG-I Ca^{2+} accumulation in regional brain microsomes [139]. In this study, brain stem and spinal cord microsomes demonstrated a higher TG-I component of total Ca^{2+} uptake than other brain regions. We explored this regional heterogeneity in pig brain and spinal cord microsomes to determine whether similar differences were also found in another mammalian species. We found that pig spinal cord microsomes contain the highest percentage of TG-I Ca^{2+}

sequestration (24.2%) as compared to other pig brain regions (Table 4). Brain stem microsomes displayed higher TG-I proportions (17.6%) than cerebellar microsomes (12.1%) which were similar to forebrain microsomes (11.7%). The similar enrichment of the TG-I pool in spinal cord and brain stem microsomes prepared from two distinct mammalian species suggests that the TG-I pool plays a prominent role in spinal cord and brain stem function.

These microsomal preparations as well as the rat whole brain homogenates and microsomes in the present study represent the contribution of a variety of different cell types. To distinguish the contribution of specific cell types to the TG-I pool, we determined TG-I Ca^{2+} accumulation in primary cultures of rat cerebellar granule cells and whole brain astrocytes. The cerebellar granule cells were found to have three times the level of TG-I Ca^{2+} accumulation as compared to the astrocytes (Table 4). These results suggest that the TG-I Ca^{2+} pool may have a unique importance for neuronal cells as compared to glial cells. Intracellular Ca^{2+} pools have been shown to play a prominent role in the survival of developing cerebellar granule neurons in cultures [149]. Granule cells display a maturation associated change in the sensitivity of their intracellular pools to TG suggesting that the TG-I Ca^{2+} pool may somehow play a role in granule cell maturation. A TG-I pool with pharmacological properties very similar to those that we have observed in our rat brain preparations has been described previously in the rat pheochromocytoma PC-12 cell line derived from the adrenal medulla [110]. The amount of Ca^{2+} trapped within the TG-I pool was shown to increase

significantly after a prolonged elevation of intracellular Ca^{2+} elicited by activation of Ca^{2+} influx. We have compared the TG-I pool in PC-12 cells with that in two neuronal cell lines and two glial cell lines derived from human brain tissues. SH-SY5Y human neuroblastoma cells display TG-I Ca^{2+} accumulation (23.1%) similar in proportions to that in PC-12 cells (29.8%) while NBFL human neuroblastoma cells had much lower proportion of TG-I Ca^{2+} accumulation (14.1%). Variations in the TG-I component of total Ca^{2+} accumulation were most obvious between the U-251 human glioblastoma cell line (4.4%) and the U-87 human glioblastoma cell line (49.1%). Since the differences in TG-I Ca^{2+} accumulation observed between different brain regions and different primary cell cultures and cancer cell lines were seen under identical assay conditions, they represent real differences in the TG-I contribution to intracellular Ca^{2+} buffering. Differences seen amongst normal tissue preparation and primary brain cell cultures presumably reflect cell and brain region-specific customization of intracellular Ca^{2+} buffering mechanisms. It is not yet clear why cancer cell lines display such widely varying proportions of the TG-I and TG-S components. Among the three neuronal cell lines examined in our study, we have noted that the relative cell growth rates (PC-12 > SH-SY5Y > NBFL) appear to correlate with the level of the TG-I Ca^{2+} accumulation. In keeping with this observation, U-251 cells are the slowest growing of all the cells studied while U-87 cells are the fastest (Watson and Verma, unpublished observations). It is conceivable that Ca^{2+} pools in highly mitotic cells have a higher TG-I component. However, this

would not explain the marked differences in TG-I Ca^{2+} accumulation seen in different regions of the adult mammalian brain.

To examine the distribution of the TG-I Ca^{2+} pool in peripheral rat tissues, we utilized a modification of the Ca^{2+} uptake assay which employs the use of fresh frozen cryostat tissue sections in Ca^{2+} uptake studies instead of homogenates or microsomes. This modification allows for not only the quantification of ER Ca^{2+} accumulation into various tissues, but also can localize the anatomical distribution of Ca^{2+} accumulating compartments to distinct tissue elements of a given organ. With this methodology, the TG-I component of total ER Ca^{2+} accumulation in different organs was determined by monitoring $^{45}Ca^{2+}$ accumulation into sections in the absence and presence of TG (10 nmol/mg protein). Brain sections displayed the highest TG-I component of total Ca^{2+} accumulation (20%) amongst all organs examined (Table 5). Adrenal gland TG-I Ca^{2+} accumulation (18.5%) was close to that seen for brain, with the heart displaying the next highest TG-I component (12.6%). Other tissues displayed intermediate levels ranging from 8.9% in the kidney to 0% in the bladder. Although brain, heart, and skeletal muscle are all electrically excitable tissues with prominent SR/ER Ca^{2+} buffering activity, only 1.7% of the skeletal muscle SR Ca^{2+} accumulation was insensitive to TG. Smooth muscle activity also requires the constant participation of ER Ca^{2+} uptake and release mechanisms. However, smooth muscle-rich tissues, such as uterus, large intestine, and bladder, demonstrated very little TG-I accumulation (5.1%, 4.8%, and 0%,

respectively). In fact, the only clear association of the TG-I pool appears to be with nervous tissues. The adrenal gland medulla is a modified sympathetic ganglion and the high proportion of TG-I Ca^{2+} accumulation in this tissue may be associated with this component (see below). What function the TG-I serves in neuronal tissues is unclear, although a clearer delineation of its distribution amongst different brain regions could provide more insight.

To more accurately localize the TG-I Ca^{2+} accumulation in rat brain and adrenal gland, we utilized tissue autoradiography of the accumulated $^{45}Ca^{2+}$ in tissue sections. In the adrenal gland, the majority of accumulated Ca^{2+} is seen in the medulla, with lower levels seen in the adrenal cortex and the zona granulosa having greater accumulation than other cortical layers (Data not shown). The TG-I component of total Ca^{2+} accumulation by adrenal gland sections is predominantly associated with the medulla, which is enriched in sympathetic neuronal chromaffin cells. This is consistent with the high levels of TG-I Ca^{2+} accumulation seen in nervous tissue preparations from rat and pig. In rat brain sections, total Ca^{2+} accumulation shows remarkable regional enrichment in specific cell layers, such as cerebral cortex, hippocampus, striatum, and cerebellar cortex. TG selectively inhibits Ca^{2+} accumulation into a number of these regions, including the cerebral cortex, hippocampus, striatum, and cerebellum (Figure 11). Many brain regions remain insensitive to TG inhibition at doses that maximally inhibit TG-S (see above). This TG-I Ca^{2+}

compartment is highly enriched in the thalamus, the superior and inferior colliculi, brain stem, pontine nuclei, and deep cerebellar nuclei. Areas demonstrating very little TG-I Ca^{2+} accumulation include the olfactory tubercle, basal forebrain, striatum, hypothalamus, and substantia nigra (Figure 11). . The cerebral cortex demonstrates both TG-S and TG-I Ca^{2+} accumulation, however, the patterning of these two compartments appears to be distinct. The TG-S component is selectively enriched in the superficial cortical layers, while TG-I is enriched in the peri-collosal, deep cerebral cortical layers. In the hippocampus, the cell layers contain both TG-S and TG-I components. In the cerebellum, very high levels of TG-S are associated with the cortical layers with highest levels in the Purkinje cell layer followed by the molecular layer. The granule cell layer and deep cerebellar nuclei display lower levels of total Ca^{2+} accumulation. The highest TG-I components of cerebellar Ca^{2+} accumulation are associated with the deep cerebellar nuclei followed by the Purkinje cell layer. Molecular and granule cell layers have lower TG-I Ca^{2+} accumulation. These results distinguish, for the first time, the novel compartmentation of TG-S and TG-I Ca^{2+} pools in rat brain. These highly unique expression patterns for the TG-I pool imply a very important regionally selective function for this process in the mammalian brain. What could be the function of the TG-I pool in these regions? In general the brain stem displays enriched TG-I Ca^{2+} accumulation, and many nuclei in this region are involved in the regulation of "vegetative" functions, such as control of breathing and heart rate. Many of these regions also are involved in regulating consciousness as components of

the reticular activating system (Figure 11). Perhaps the regulation of intracellular Ca^{2+} in neuronal cells in these structures requires distinct buffering mechansims than those of other structures. These unique requirements may include the lack of sensitivity to Ca^{2+} mobilizing agents, faster Ca^{2+} accumulation rates under physiological anionic conditions and insensitivity to redox modification. Another possibility is suggested by the enrichment of TG-I pool in brain structures which integrate a large number of neuronal inputs. For example, spinal cord and brain stem structures receive rich innervation from peripheral as well as central sites. The deep cerebellar nuclei receives the major output of the cerebellar cortex via the Purkinje cells. Likewise, the inferior and superior colliculi are major synaptic relay stations for visual and auditory information, while all sensory input to the cerebral cortex proceeds via synaptic relays in the thalamic nuclei. Perhaps regions of high synaptic input require unique Ca^{2+} buffering compartments, accurate regulation of neuronal Ca^{2+} levels are paramount for the maintenance of synaptic activity. Clearly, a lot more work needs to be done to understand the role of the TG-I Ca^{2+} compartment. While our fanciful speculations attempt to synthesize functions for this compartment based on what we have learned about it in our studies, we hope that our characterization of this pool may someday shed important light on the role of Ca^{2+} in the central nervous system.

TG selectively inhibits Ca^{2+} accumulation into a number of these regions, including: cerebral cortex, hippocampus, striatum, and cerebellum. Certain brain regions, however, are relatively insensitive to TG inhibition at doses that maximally inhibit TG-S (see above). Ca^{2+} accumulation in these regions thus represents the TG-I Ca^{2+} pool. This compartment is highly enriched in the thalamus, the superior and inferior colliculi, brain stem, pontine nuclei, and deep cerebellar nuclei. Areas demonstrating very little TG-I Ca^{2+} accumulation include: striatum, hypothalamus, substantia nigra, olfactory tubercle, and basal forebrain. While the cerebral cortex demonstrates both TG-S and TG-I Ca^{2+} accumulation, the patterning of these two processes appears to be distinct. Thus, the TG-S component is selectively enriched in the superficial cortical layers, and TG-I is enriched in the peri-collosal, deep cerebral cortical layers. In the hippocampus, TG-S is highly concentrated in the pyramidal and dentate gyrus cell layers, while the TG-I shows a more diffuse distribution not associated with cell bodies of these layers. In the cerebellum, very high levels of TG-S are associated with the cortical layers with highest levels in the Purkinje cell layer followed by the molecular layer. The granule cell layer and deep cerebellar nuclei display lower levels of total Ca^{2+} accumulation. The highest TG-I components of cerebellar Ca^{2+} accumulation are associated with the deep cerebellar nuclei followed by the Purkinje cell layer. Molecular and granule cell layers have lower TG-I Ca^{2+} accumulation. These results reveal, for the first time, a novel compartmentation of TG-S and TG-I Ca^{2+} pools in rat brain. The highly unique expression patterns for the TG-I pool observed in our

studies imply a very important regionally selective function for this process in the mammalian brain.

REFERENCES:

1 Aalkjaer, C. and Mulvany, M.J., Effect of ouabain on tone, membrane potential and sodium efflux compared with [3H]ouabain binding in rat resistance vessels, J Physiol (Lond), 362 (1985) 215-31.

2 Adams, M.H., Ostrosky, J.D. and Kirkwood, C.F., Therapeutic evaluation of omeprazole, Clin Pharm, 7 (1988) 725-45.

3 Alford, S., Frenguelli, B.G., Schofield, J.G. and Collingridge, G.L., Characterization of Ca2+ signals induced in hippocampal CA1 neurones by the synaptic activation of NMDA receptors, J Physiol (Lond), 469 (1993) 693-716.

4 Amzel, L.M. and Pedersen, P.L., Proton atpases: structure and mechanism, Annu Rev Biochem, 52 (1983) 801-24.

5 Andersen, J.P., Monomer-oligomer equilibrium of sarcoplasmic reticulum Ca-ATPase and the role of subunit interaction in the Ca2+ pump mechanism, Biochim Biophys Acta, 988 (1989) 47-72.

6 Andersen, J.P. and Vilsen, B., Primary ion pumps, Curr Opin Cell Biol, 2 (1990) 722-30.

7 Armstrong, R.C., Isolation and characterization of immature oligodendrocyte lineage cells, Methods, 16 (1998) 282-92.

8 Barish, M.E., Increases in intracellular calcium ion concentration during depolarization of cultured embryonic Xenopus spinal neurones, J Physiol (Lond), 444 (1991) 545-65.

9 Beil, W., Staar, U. and Sewing, K.F., Studies on the mechanism of action of the omeprazole-derived cyclic sulphenamide, Biochem Pharmacol, 37 (1988) 843-8.

10 Berridge, M.J., Calcium oscillations, J Biol Chem, 265 (1990) 9583-6.

11 Berridge, M.J., Neuronal calcium signaling, Neuron, 21 (1998) 13-26.

12 Berridge, M.J. and Galione, A., Cytosolic calcium oscillators, Faseb J, 2 (1988) 3074-82.

13 Berridge, M.J. and Irvine, R.F., Inositol phosphates and cell signalling, Nature, 341 (1989) 197-205.

14 Bian, X., Hughes, F.M., Jr., Huang, Y., Cidlowski, J.A. and Putney, J.W., Jr., Roles of cytoplasmic Ca2+ and intracellular Ca2+ stores in induction and suppression of apoptosis in S49 cells, Am J Physiol, 272 (1997) C1241-9.

15 Boobis, A.R., Fawthrop, D.J. and Davies, D.S., Mechanisms of cell death, Trends Pharmacol Sci, 10 (1989) 275-80.

16 Brandl, C.J., deLeon, S., Martin, D.R. and MacLennan, D.H., Adult forms of the Ca2+ATPase of sarcoplasmic reticulum. Expression in developing skeletal muscle, J Biol Chem, 262 (1987) 3768-74.

17 Brodin, P., Falchetto, R., Vorherr, T. and Carafoli, E., Identification of two domains which mediate the binding of activating phospholipids to the plasma-membrane Ca2+ pump, Eur J Biochem, 204 (1992) 939-46.

18 Burk, S.E., Lytton, J., MacLennan, D.H. and Shull, G.E., cDNA cloning, functional expression, and mRNA tissue distribution of a third organellar Ca2+ pump, J Biol Chem, 264 (1989) 18561-8.

19 Campbell, A.M., Kessler, P.D., Sagara, Y., Inesi, G. and Fambrough, D.M., Nucleotide sequences of avian cardiac and brain SR/ER Ca(2+)-ATPases and functional comparisons with fast twitch Ca(2+)-ATPase. Calcium affinities and inhibitor effects, J Biol Chem, 266 (1991) 16050-5.

20 Carafoli, E., Intracellular calcium homeostasis, Annu Rev Biochem, 56 (1987) 395-433.

21 Caroni, P. and Carafoli, E., The Ca2+-pumping ATPase of heart sarcolemma. Characterization, calmodulin dependence, and partial purification, J Biol Chem, 256 (1981) 3263-70.

22 Caroni, P. and Carafoli, E., Regulation of Ca2+-pumping ATPase of heart sarcolemma by a phosphorylation-dephosphorylation Process, J Biol Chem, 256 (1981) 9371-3.

23 Choi, H.S. and Eisner, D.A., The effects of inhibition of the sarcolemmal Ca-ATPase on systolic calcium fluxes and intracellular calcium concentration in rat ventricular myocytes, Pflugers Arch, 437 (1999) 966-71.

24 Chuang, D.M., Neurotransmitter receptors and phosphoinositide turnover, Annu Rev Pharmacol Toxicol, 29 (1989) 71-110.

25 Clarke, D.M., Loo, T.W., Inesi, G. and MacLennan, D.H., Location of high affinity Ca2+-binding sites within the predicted transmembrane domain of the sarcoplasmic reticulum Ca2+-ATPase, Nature, 339 (1989) 476-8.

26 Cohen, A.S., Moore, K.A., Bangalore, R., Jafri, M.S., Weinreich, D. and Kao, J.P., Ca(2+)-induced Ca2+ release mediates Ca2+ transients evoked by single action potentials in rabbit vagal afferent neurones, J Physiol (Lond), 499 (1997) 315-28.

27 Darby, P.J., Kwan, C.Y. and Daniel, E.E., Selective inhibition of oxalate-stimulated Ca2+ transport by cyclopiazonic acid and thapsigargin in smooth muscle microsomes, Can J Physiol Pharmacol, 74 (1996) 182-92.

28 Davidson, G.A. and Varhol, R.J., Kinetics of thapsigargin-Ca(2+)-ATPase (sarcoplasmic reticulum) interaction reveals a two-step binding mechanism and picomolar inhibition, J Biol Chem, 270 (1995) 11731-4.

29 de Meis, L. and Vianna, A.L., Energy interconversion by the Ca2+-dependent ATPase of the sarcoplasmic reticulum, Annu Rev Biochem, 48 (1979) 275-92.

30 de Mendonca, R.L., Beck, E., Rumjanek, F.D. and Goffeau, A., Cloning and characterization of a putative calcium-transporting ATPase gene from Schistosoma mansoni, Mol Biochem Parasitol, 72 (1995) 129-39.

31 Dilman, W.H., Regulation of expression of cardiac sarcoplasmic reticulum proteins under pathophysiological conditions, Mol Cell Biochem, 157 (1996) 125-128.

32 D'Mello, S.R., Galli, C., Ciotti, T. and Calissano, P., Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP, Proc Natl Acad Sci U S A, 90 (1993) 10989-93.

33 Dode, L., Wuytack, F., Kools, P.F., Baba-Aissa, F., Raeymaekers, L., Brike, F., van de Ven, W.J., Casteels, R. and Brik, F., cDNA cloning, expression and chromosomal localization of the human sarco/endoplasmic reticulum Ca(2+)-ATPase 3 gene [published erratum appears in Biochem J 1996 Nov 1;319(Pt 3):1008], Biochem J, 318 (1996) 689-99.

34 Dolor, R.J., Hurwitz, L.M., Mirza, Z., Strauss, H.C. and Whorton, A.R., Regulation of extracellular calcium entry in endothelial cells: role of intracellular calcium pool, Am J Physiol, 262 (1992) C171-81.

35 Favre, C.J., Schrenzel, J., Jacquet, J., Lew, D.P. and Krause, K.H., Highly supralinear feedback inhibition of Ca2+ uptake by the Ca2+ load of intracellular stores, J Biol Chem, 271 (1996) 14925-30.

36 Feher, J.J. and Lipford, G.B., Mechanism of action of ryanodine on cardiac sarcoplasmic reticulum, Biochim Biophys Acta, 813 (1985) 77-86.

37 Ferris, C.D., Huganir, R.L., Supattapone, S. and Snyder, S.H., Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles, Nature, 342 (1989) 87-9.

38 Fitzsimmons, T.J., McRoberts, J.A., Tachiki, K.H. and Pandol, S.J., Acyl-coenzyme A causes Ca2+ release in pancreatic acinar cells, J Biol Chem, 272 (1997) 31435-40.

39 Foskett, J.K. and Wong, D., Free cytoplasmic Ca2+ concentration oscillations in thapsigargin- treated parotid acinar cells are caffeine- and ryanodine-sensitive, J Biol Chem, 266 (1991) 14535-8.

40 Friel, D.D. and Tsien, R.W., A caffeine- and ryanodine-sensitive Ca2+ store in bullfrog sympathetic neurones modulates effects of Ca2+ entry on [Ca2+]i, J Physiol (Lond), 450 (1992) 217-46.

41 Furuichi, T. and Mikoshiba, K., Inositol 1, 4, 5-trisphosphate receptor-mediated Ca2+ signaling in the brain, J Neurochem, 64 (1995) 953-60.

42 Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K., Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400, Nature, 342 (1989) 32-8.

43 Galione, A., Cyclic ADP-ribose, the ADP-ribosyl cyclase pathway and calcium signalling, Mol Cell Endocrinol, 98 (1994) 125-31.

44 Gallo, V., Kingsbury, A., Balazs, R. and Jorgensen, O.S., The role of depolarization in the survival and differentiation of cerebellar granule cells in culture, J Neurosci, 7 (1987) 2203-13.

45 Genazzani, A.A. and Galione, A., Nicotinic acid-adenine dinucleotide phosphate mobilizes Ca2+ from a thapsigargin-insensitive pool, Biochem J, 315 (1996) 721-5.

46 Ghosh, T.K., Bian, J.H., Short, A.D., Rybak, S.L. and Gill, D.L., Persistent intracellular calcium pool depletion by thapsigargin and its influence on cell growth, J Biol Chem, 266 (1991) 24690-7.

47 Goffeau, A. and Slayman, C.W., The proton-translocating ATPase of the fungal plasma membrane, Biochim Biophys Acta, 639 (1981) 197-223.

48 Gould, G.W., East, J.M., Froud, R.J., McWhirter, J.M., Stefanova, H.I. and Lee, A.G., A kinetic model for the Ca2+ + Mg2+-activated ATPase of sarcoplasmic reticulum, Biochem J, 237 (1986) 217-27.

49 Gutheil, J.C., Hart, S.R., Belani, C.P., Melera, P.W. and Hussain, A., Alterations in Ca2+ transport ATPase and P-glycoprotein expression can mediate resistance to thapsigargin, J Biol Chem, 269 (1994) 7976-81.

50 Hardy, S.J., Robinson, B.S., Ferrante, A., Hii, C.S., Johnson, D.W., Poulos, A. and Murray, A.W., Polyenoic very-long-chain fatty acids mobilize intracellular calcium from a thapsigargin-insensitive pool in human neutrophils. The relationship between Ca2+ mobilization and superoxide production induced by long- and very-long-chain fatty acids, Biochem J, 311 (1995) 689-97.

51 Harigaya, S. and Schwartz, A., Rate of calcium binding and uptake in normal animal and failing human cardiac muscle, Cir Res, 25 (1969) 781-794.

52 Hasselbach, W., Relaxing factor and the relaxation of muscle. In J.A.V. Butler and H.E. Huxley (Eds.), Prog in Biophys, Vol. 14, Pergamon Press Ltd., New York, 1964, pp. 169-222.

53 Hatefi, Y., The mitochondrial electron transport and oxidative phosphorylation system, Annu Rev Biochem, 54 (1985) 1015-69.

54 Hauser, K., Pavlovic, N., Kissmehl, R. and Plattner, H., Molecular characterization of a sarco(endo)plasmic reticulum Ca2+- ATPase gene from Paramecium tetraurelia and localization of its gene product to sub-plasmalemmal calcium stores, Biochem J, 334 (1998) 31-8.

55 He, H., Lam, M., McCormick, T.S. and Distelhorst, C.W., Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl- 2, J Cell Biol, 138 (1997) 1219-28.

56 Henzi, V. and MacDermott, A.B., Characteristics and function of Ca(2+)- and inositol 1,4,5- trisphosphate-releasable stores of Ca2+ in neurons, Neuroscience, 46 (1992) 251-73.

57 Hernandez-Cruz, A., Escobar, A.L. and Jimenez, N., Ca(2+)-induced Ca2+ release phenomena in mammalian sympathetic neurons are critically dependent on the rate of rise of trigger Ca2+, J Gen Physiol, 109 (1997) 147-67.

58 Hirono, M., Takamura, K., Ito, Y., Nakano, Y., Chikaoka, Y., Suzuki, N. and Yoshioka, T., Role of Ca(2+)-ATPase in spontaneous oscillations of cytosolic free Ca2+ in GH3 rat pituitary cells [In Process Citation], Cell Calcium, 25 (1999) 125-35.

59 Hua, S.Y., Tokimasa, T., Takasawa, S., Furuya, Y., Nohmi, M., Okamoto, H. and Kuba, K., Cyclic ADP-ribose modulates Ca2+ release channels for activation by physiological Ca2+ entry in bullfrog sympathetic neurons, Neuron, 12 (1994) 1073-9.

60 Hutchins, J.B. and Barger, S.W., Why neurons die: cell death in the nervous system, Anat Rec, 253 (1998) 79-90.

61 Ikemoto, N., Sreter, F.A., Nakamura, A. and Gergely, J., Tryptic digestion and localization of calcium uptake and ATPase activity in tragments of sarcoplasmic reticulum, J Ultrastruct Res, 23 (1968) 216-32.

62 Inesi, G. and Kirtley, M.R., Structural features of cation transport ATPases, J Bioenerg Biomembr, 24 (1992) 271-83.

63 Inui, M., Saito, A. and Fleischer, S., Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures, J Biol Chem, 262 (1987) 15637-42.

64 Islam, M.S., Rorsman, P. and Berggren, P.O., Ca(2+)-induced Ca2+ release in insulin-secreting cells, FEBS Lett, 296 (1992) 287-91.

65 Jacobs, J.M. and Meyer, T., Control of action potential-induced Ca2+ signaling in the soma of hippocampal neurons by Ca2+ release from intracellular stores, J Neurosci, 17 (1997) 4129-35.

66 Jorgensen, M.B., Johansen, F.F. and Diemer, N.H., Removal of the entorhinal cortex protects hippocampal CA-1 neurons from ischemic damage, Acta Neuropathol, 73 (1987) 189-94.

67 Kaneko, Y. and Tsukamoto, A., Thapsigargin-induced persistent intracellular calcium pool depletion and apoptosis in human hepatoma cells, Cancer Lett, 79 (1994) 147-55.

68 Karnushina, I., Suzuki, R., Padgett, W. and Daly, J.W., Degeneration of CA1 neurons in hippocampus after ischemia in Mongolian gerbils: cyclic AMP-systems, Brain Res, 268 (1983) 87-94.

69 Kass, G.E. and Orrenius, S., Calcium Signaling and Cytotoxicity, Environ Health Perspect, 107 Suppl 1 (1999) 25-35.

70 Kennedy, B.G. and Mangini, N.J., Plasma membrane calcium-ATPase in cultured human retinal pigment epithelium, Exp Eye Res, 63 (1996) 547-56.

71 Kiessling, M., Stumm, G., Xie, Y., Herdegen, T., Aguzzi, A., Bravo, R. and Gass, P., Differential transcription and translation of immediate early genes in the gerbil hippocampus after transient global ischemia, J Cereb Blood Flow Metab, 13 (1993) 914-24.

72 Kissmehl, R., Huber, S., Kottwitz, B., Hauser, K. and Plattner, H., Subplasmalemmal Ca-stores in Paramecium tetraurelia. Identification and characterisation of a sarco(endo)plasmic reticulum-like Ca(2+)-ATPase by phosphoenzyme intermediate formation and its inhibition by caffeine, Cell Calcium, 24 (1998) 193-203.

73 Kostyuk, P. and Verkhratsky, A., Calcium stores in neurons and glia, Neuroscience, 63 (1994) 381-404.

74 Kramer, R., Mitochondrial carrier proteins can reversibly change their transport mode: the cases of the aspartate/glutamate and the phosphate carrier, Exp Physiol, 83 (1998) 259-65.

75 Kristian, T. and Siesjo, B.K., Calcium in ischemic cell death, Stroke, 29 (1998) 705-18.

76 Kristian, T. and Siesjo, B.K., Calcium-related damage in ischemia, Life Sci, 59 (1996) 357-67.

77 Kruman, II and Mattson, M.P., Pivotal role of mitochondrial calcium uptake in neural cell apoptosis and necrosis, J Neurochem, 72 (1999) 529-40.

78 Kuznetsov, G., Brostrom, M.A. and Brostrom, C.O., Demonstration of a calcium requirement for secretory protein processing and export, JBC, 267 (1992) 3932-3939.

79 Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R.L. and Distelhorst, C.W., Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes, Proc Natl Acad Sci U S A, 91 (1994) 6569-73.

80 Lee, H.C., Aarhus, R. and Graeff, R.M., Sensitization of calcium-induced calcium release by cyclic ADP-ribose and calmodulin, J Biol Chem, 270 (1995) 9060-6.

81 Lipscombe, D., Madison, D.V., Poenie, M., Reuter, H., Tsien, R.W. and Tsien, R.Y., Imaging of cytosolic Ca2+ transients arising from Ca2+ stores and Ca2+ channels in sympathetic neurons, Neuron, 1 (1988) 355-65.

82 Llano, I., DiPolo, R. and Marty, A., Calcium-induced calcium release in cerebellar Purkinje cells, Neuron, 12 (1994) 663-73.

83 Lytton, J., Westlin, M. and Hanley, M.R., Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca- ATPase family of calcium pumps, J Biol Chem, 266 (1991) 17067-71.

84 Lytton, J., Zarain-Herzberg, A., Periasamy, M. and MacLennan, D.H., Molecular cloning of the mammalian smooth muscle sarco(endo)plasmic reticulum Ca2+-ATPase, J Biol Chem, 264 (1989) 7059-65.

85 Ma, H., Zhong, L., Inesi, G., Fortea, I., Soler, F. and Fernandez-Belda, F., Overlapping effects of S3 stalk segment mutations on the affinity of Ca2+-ATPase (SERCA) for thapsigargin and cyclopiazonic acid, Biochemistry, 38 (1999) 15522-7.

86 Macara, I.G., Kustin, K. and Cantley, L.C., Jr., Glutathione reduces cytoplasmic vanadate. Mechanism and physiological implications, Biochim Biophys Acta, 629 (1980) 95-106.

87 Magun, B.E. and Rodland, K.D., Transient inhibition of protein synthesis induces the immediate early gene VL30: alternative mechanism for thapsigargin-induced gene expression, Cell Growth Differ, 6 (1995) 891-7.

88 Maloney, P.C. and Wilson, T.H., The evolution of ion pumps, Bioscience, 35 (1985) 43-48.

89 Martone, M.E., Alba, S.A., Edelman, V.M., Airey, J.A. and Ellisman, M.H., Distribution of inositol-1,4,5-trisphosphate and ryanodine receptors in rat neostriatum, Brain Res, 756 (1997) 9-21.

90 Mason, M.J., Garcia-Rodriguez, C. and Grinstein, S., Coupling between intracellular Ca2+ stores and the Ca2+ permeability of the plasma membrane. Comparison of the effects of thapsigargin, 2,5-di- (tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes, J Biol Chem, 266 (1991) 20856-62.

91 McQueen, D.S. and Ribeiro, J.A., Effects of ouabain on carotid body chemoreceptor activity in the cat, J Physiol (Lond), 335 (1983) 221-35.

92 Mears, D., Leighton, X., Atwater, I. and Rojas, E., Tetracaine stimulates insulin secretion from the pancreatic beta-cell by release of intracellular calcium, Cell Calcium, 25 (1999) 59-68.

93 Meissner, G. and Allen, R., Evidence for two types of rat liver microsomes with differing permeability to glucose and other small molecules, J Biol Chem, 256 (1981) 6413-22.

94 Mennel, H.D., Sauer, D., Rossberg, C., Bielenberg, G.W. and Krieglstein, J., Morphology of tissue damage due to experimental cerebral ischemia in rats, Exp Pathol, 35 (1988) 219-30.

95 Meyer, T. and Stryer, L., Transient calcium release induced by successive increments of inositol 1,4,5-trisphosphate, Proc Natl Acad Sci U S A, 87 (1990) 3841-5.

96 Meyer, T., Wensel, T. and Stryer, L., Kinetics of calcium channel opening by inositol 1,4,5-trisphosphate, Biochemistry, 29 (1990) 32-7.

97 Mignery, G.A., Sudhof, T.C., Takei, K. and De Camilli, P., Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor, Nature, 342 (1989) 192-5.

98 Miller, K.K., Verma, A., Snyder, S.H. and Ross, C.A., Localization of an endoplasmic reticulum calcium ATPase mRNA in rat brain by in situ hybridization, Neuroscience, 43 (1991) 1-9.

99 Milner, R.E., Famulski, K.S. and Michalak, M., Calcium binding proteins in the sarcoplasmic/endoplasmic reticulum of muscle and nonmuscle cells, Mol Cell Biochem, 112 (1992) 1-13.

100 Nath, R., Raser, K.J., Hajimohammadreza, I. and Wang, K.K., Thapsigargin induces apoptosis in SH-SY5Y neuroblastoma cells and cerebrocortical cultures, Biochem Mol Biol Int, 43 (1997) 197-205.

101 Nath, R., Raser, K.J., Stafford, D., Hajimohammadreza, I., Posner, A., Allen, H., Talanian, R.V., Yuen, P., Gilbertsen, R.B. and Wang, K.K., Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis, Biochem J, 319 (1996) 683-90.

102 Ng, L.T., Selwyn, M.J. and Choo, H.L., Inorganic phosphate is the major component of the thermostable cytoplasmic fraction which stimulates mitochondrial anion uniport, Biochim Biophys Acta, 2 (1993) 180-4.

103 Nigam, S.K., Goldberg, A.L., Ho, S., Rohde, M.F., Bush, K.T. and Sherman, M., A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes Ca(2+)-binding proteins and members of the thioredoxin superfamily, J Biol Chem, 269 (1994) 1744-9.

104 Norregaard, A., Vilsen, B. and Andersen, J.P., Transmembrane segment M3 is essential to thapsigargin sensitivity of the sarcoplasmic reticulum Ca(2+)-ATPase, J Biol Chem, 269 (1994) 26598-601.

105 Palade, P., Drug-induced Ca2+ release from isolated sarcoplasmic reticulum. III. Block of Ca2+-induced Ca2+ release by organic polyamines, J Biol Chem, 262 (1987) 6149-54.

106 Palade, P., Mitchell, R.D. and Fleischer, S., Spontaneous calcium release from sarcoplasmic reticulum. General description and effects of calcium, J Biol Chem, 258 (1983) 8098-107.

107 Paschen, W., Disturbances of calcium homeostasis within the endoplasmic reticulum may contribute to the development of ischemic-cell damage, Med Hypotheses, 47 (1996) 283-8.

108 Passos, A.P. and Garcia, C.R., Characterization of Ca2+ transport activity associated with a non- mitochondrial calcium pool in the rodent malaria parasite P. chabaudi, Biochem Mol Biol Int, 42 (1997) 919-25.

109 Pedersen, P.L. and Carafoli, E., Ion motive ATPases. Part I. Ubiquity, properties, and significance to cell function, TIBS, 12 (1987) 146-150.

110 Pizzo, P., Fasolato, C. and Pozzan, T., Dynamic properties of an inositol 1,4,5-trisphosphate- and thapsigargin- insensitive calcium pool in mammalian cell lines, J Cell Biol, 136 (1997) 355-66.

111 Preston, G.A., Barrett, J.C., Biermann, J.A. and Murphy, E., Effects of alterations in calcium homeostasis on apoptosis during neoplastic progression, Cancer Res, 57 (1997) 537-42.

112 Qian, Z., Okuhara, D., Abe, M.K. and Rosner, M.R., Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel, J Biol Chem, 274 (1999) 1621-7.

113 Rooney, E. and Meldolesi, J., The endoplasmic reticulum in PC12 cells. Evidence for a mosaic of domains differently specialized in Ca2+ handling, J Biol Chem, 271 (1996) 29304-11.

114 Ross, C.A., Meldolesi, J., Milner, T.A., Satoh, T., Supattapone, S. and Snyder, S.H., Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje neurons, Nature, 339 (1989) 468-70.

115 Sagara, Y., Fernandez-Belda, F., de Meis, L. and Inesi, G., Characterization of the inhibition of intracellular Ca2+ transport ATPases by thapsigargin, J Biol Chem, 267 (1992) 12606-13.

116 Salvador, J.M. and Mata, A.M., Characterization of the intracellular and the plasma membrane Ca2+- ATPases in fractionated pig brain membranes using calcium pump inhibitors, Arch Biochem Biophys, 351 (1998) 272-8.

117 Sambrook, J.F., The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum, Cell, 61 (1990) 197-9.

118 Schanne, F.A., Kane, A.B., Young, E.E. and Farber, J.L., Calcium dependence of toxic cell death: a final common pathway, Science, 206 (1979) 700-2.

119 Senior, A.E. and Wise, J.G., The proton-ATPase of bacteria and mitochondria, J Membr Biol, 73 (1983) 105-24.

120 Seymour-Laurent, K.J. and Barish, M.E., Inositol 1,4,5-trisphosphate and ryanodine receptor distributions and patterns of acetylcholine- and caffeine-induced calcium release in cultured mouse hippocampal neurons, J Neurosci, 15 (1995) 2592-608.

121 Sharp, A.H., McPherson, P.S., Dawson, T.M., Aoki, C., Campbell, K.P. and Snyder, S.H., Differential immunohistochemical localization of inositol 1,4,5-trisphosphate- and ryanodine-sensitive Ca2+ release channels in rat brain, J Neurosci, 13 (1993) 3051-63.

122 Shmigol, A., Verkhratsky, A. and Isenberg, G., Calcium-induced calcium release in rat sensory neurons, J Physiol (Lond), 489 (1995) 627-36.

123 Simpson, P.B., Challiss, R.A. and Nahorski, S.R., Neuronal Ca2+ stores: activation and function, Trends Neurosci, 18 (1995) 299-306.

124 Simpson, P.B. and Russell, J.T., Role of sarcoplasmic/endoplasmic-reticulum Ca2+-ATPases in mediating Ca2+ waves and local Ca2+-release microdomains in cultured glia, Biochem J, 325 (1997) 239-47.

125 Slinchenko, N.N., Bratkova, N.F., Kosterin, S.A., Zimina, V.P. and Chernysh, I.G., Effects of eosin Y on the catalytic and functional activities of Mg2+,ATP-dependent calcium pump of smooth muscle cell plasma membrane, Biochemistry (Mosc), 63 (1998) 685-90.

126 Stefanova, H.I., Jane, S.D., East, J.M. and Lee, A.G., Effects of Mg2+ and ATP on the phosphate transporter of sarcoplasmic reticulum, Biochim Biophys Acta, 1064 (1991) 329-34.

127 Stojilkovic, S.S. and Catt, K.J., Calcium oscillations in anterior pituitary cells, Endocr Rev, 13 (1992) 256-80.

128 Supattapone, S., Worley, P.F., Baraban, J.M. and Snyder, S.H., Solubilization, purification, and characterization of an inositol trisphosphate receptor, J Biol Chem, 263 (1988) 1530-4.

129 Tanaka, K., Tsukahara, T., Hashimoto, N., Ogata, N., Yonekawa, Y., Kimura, T. and Taniguchi, T., Effect of nerve growth factor on delayed neuronal death after cerebral ischaemia, Acta Neurochir, 129 (1994) 64-71.

130 Tanaka, Y. and Tashjian, A.H., Jr., Functional identification and quantitation of three intracellular calcium pools in GH4C1 cells: evidence that the

caffeine-responsive pool is coupled to a thapsigargin-resistant, ATP-dependent process, Biochemistry, 32 (1993) 12062-73.

131 Taylor, R.S., Jones, S.M., Dahl, R.H., Nordeen, M.H. and Howell, K.E., Characterization of the Golgi complex cleared of proteins in transit and examination of calcium uptake activities, Mol Biol Cell, 8 (1997) 1911-31.

132 Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P., Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase, Proc Natl Acad Sci U S A, 87 (1990) 2466-70.

133 Treiman, M., Caspersen, C. and Christensen, S.B., A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca(2+)-ATPases, Trends Pharmacol Sci, 19 (1998) 131-5.

134 Trotta, E.E. and de Meis, L., Adenosine 5'-triphosphate-orthophosphate exchange catalyzed by the Ca2+- transport ATPase of brain. Activation by a small transmembrane Ca2+ gradient, J Biol Chem, 253 (1978) 7821-5.

135 Tsukamoto, A. and Kaneko, Y., Thapsigargin, a Ca(2+)-ATPase inhibitor, depletes the intracellular Ca2+ pool and induces apoptosis in human hepatoma cells, Cell Biol Int, 17 (1993) 969-70.

136 Usachev, Y.M. and Thayer, S.A., All-or-none Ca2+ release from intracellular stores triggered by Ca2+ influx through voltage-gated Ca2+ channels in rat sensory neurons, J Neurosci, 17 (1997) 7404-14.

137 Verboomen, H., Wuytack, F., De Smedt, H., Himpens, B. and Casteels, R., Functional difference between SERCA2a and SERCA2b Ca2+ pumps and their modulation by phospholamban, Biochem J, 286 (1992) 591-5.

138 Verkhratsky, A. and Shmigol, A., Calcium-induced calcium release in neurones, Cell Calcium, 19 (1996) 1-14.

139 Verma, A., Hirsch, D.J., Hanley, M.R., Thastrup, O., Christensen, S.B. and Snyder, S.H., Inositol trisphosphate and thapsigargin discriminate endoplasmic reticulum stores of calcium in rat brain, Biochem Biophys Res Commun, 172 (1990) 811-6.

140 Verma, A., Hirsch, D.J. and Snyder, S.H., Calcium pools mobilized by calcium or inositol 1,4,5-trisphosphate are differentially localized in rat heart and brain, Mol Biol Cell, 3 (1992) 621-31.

141 Verma, A., Ross, C.A., Verma, D., Supattapone, S. and Snyder, S.H., Rat brain endoplasmic reticulum calcium pools are anatomically and functionally segregated, Cell Regul, 1 (1990) 781-90.

142 Vilsen, B., Structure-function relationships in the Ca(2+)-ATPase of sarcoplasmic reticulum studied by use of the substrate analogue CrATP and site-directed mutagenesis. Comparison with the Na+,K(+)-ATPase, Acta Physiol Scand Suppl, 624 (1995) 1-146.

143 Waldron, R.T., Short, A.D. and Gill, D.L., Store-operated Ca2+ entry and coupling to Ca2+ pool depletion in thapsigargin-resistant cells, J Biol Chem, 272 (1997) 6440-7.

144 Wong, W.L., Brostrom, M.A., Kuznetsov, G., Gmitter-Yellen, D. and Brostrom, C.O., Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells, Biochem J, 289 (1993) 71-9.

145 Worley, P.F., Baraban, J.M. and Snyder, S.H., Inositol 1,4,5-trisphosphate receptor binding: autoradiographic localization in rat brain, J Neurosci, 9 (1989) 339-46.

146 Wu, K.-D., Lee, W.-S., Wey, J., Bungard, D. and Lytton, J., Localization and quantitation of endoplasmic reticulum Ca2+-ATPase isoform transcripts, Am J Physiol, 269 (1995) C775-C784.

147 Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K.S. and Casteels, R., A sarco/endoplasmic reticulum Ca(2+)-ATPase 3-type Ca2+ pump is expressed in platelets, in lymphoid cells, and in mast cells [published erratum appears in J Biol Chem 1994 Apr 29;269(17):13056], J Biol Chem, 269 (1994) 1410-6.

148 Yamaki, H., Morita, K., Kitayama, S., Imai, Y., Itadani, K., Akagawa, Y. and Dohi, T., Cyclic ADP-ribose induces Ca2+ release from caffeine-insensitive Ca2+ pools in canine salivary gland cells, J Dent Res, 77 (1998) 1807-16.

149 Yao, C.J., Lin, C.W. and Lin-Shiau, S.Y., Roles of thapsigargin-sensitive Ca2+ stores in the survival of developing cultured neurons [In Process Citation], J Neurochem, 73 (1999) 457-65.

150 Zaidi, A. and Michaelis, M.L., Effects of reactive oxygen species on brain synaptic plasma membrane Ca(2+)-ATPase, Free Radic Biol Med, 27 (1999) 810-21.

151 Zhong, L. and Inesi, G., Role of the S3 stalk segment in the thapsigargin concentration dependence of sarco-endoplasmic reticulum Ca2+ ATPase inhibition, J Biol Chem, 273 (1998) 12994-8.

152 Zhou, Y.P., Teng, D., Dralyuk, F., Ostrega, D., Roe, M.W., Philipson, L. and Polonsky, K.S., Apoptosis in insulin-secreting cells. Evidence for the role of intracellular Ca2+ stores and arachidonic acid metabolism, J Clin Invest, 101 (1998) 1623-32.